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AT THE
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MYSORE CITY
INDIA

People's Book

INDUSTRIAL FERMENTATIONS

EDITED BY

LELAND A. UNDERKOFLER, PH.D., D.SC.
Professor, Chemistry Department
Iowa State College, Ames, Iowa

AND

RICHARD J. HICKEY, PH.D.
Research Microbiological Chemist
Commercial Solvents Corporation
Terre Haute, Indiana

VOLUME II

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CONTRIBUTORS TO VOLUME II

swell, Chief, Illinois State Water Survey, Urbana, Ill.

ghill, Director of Research, Abbott Laboratories, North go, Ill.

key, Research Microbiological Chemist, Commercial Sol- Corporation, Terre Haute, Ind.*

gerheide, Research Department, Pabst Brewing Company, aukee, Wisc.

lingham, Director, Prairie Regional Laboratory, National rch Council of Canada, Saskatoon, Sask.

ndegren, Chairman, Department of Microbiology, and tor, Biological Research Laboratory, Southern Illinois ersity, Carbondale, Ill.

kwood, Microbiologist, Miles-Ames Research Laboratory, rt, Ind.

F. McCoy, Professor of Agricultural Bacteriology, Uni- of Wisconsin, Madison, Wisc.

Daniel, Microbiologist, Merck & Co., Inc., Rahway, N. J.

sh, Senior Fermentation Chemist, Prairie Regional Lab- y, National Research Council of Canada, Saskatoon, Sask.

address, University of Pennsylvania, Philadelphia 4, Pa.

Mary S. Peterson, University of Wisconsin, Madison, Wisc.

W. H. Peterson, Professor of Biochemistry, University of Wisconsin, Madison, Wisc.

A. R. Stanley, Microbiologist, The Ralph M. Parsons Company, Facilities Operation Division, Braddock Heights, Md.

J. C. Sylvester, Manager, Fermentation Research, Abbott Laboratories, North Chicago, Ill.

L. A. Underkofler, Professor of Chemistry, Iowa State College, Ames, Iowa.

J. M. Van Lanen, Assistant Director of Research, Hiram Walker & Sons, Inc., Peoria, Ill.

R. H. Vaughn, Associate Professor, Food Technology Division, University of California, Davis, Calif.

H. B. Woodruff, Assistant Director and Head of Research Section, Department of Microbiology, Merck & Co., Inc., Rahway, N. J.

FOREWORD

Plans for an authoritative compilation of modern industrial practices in the fermentation industries were originally made about 1948. Because the field is so diversified, assistance of individuals with special experience was solicited to contribute chapters relating to the different fermentations. Many unavoidable delays occurred between inception of the idea and collection of all the chapter manuscripts. When all the chapters had been assembled, the bulk of the manuscript was such that it became necessary to publish in two volumes.

All the contributors have generously given most valuable assistance, some in the face of overwork and ill health. They have felt with the editors the disappointments attendant on the various delays that have occurred between first writing and actual publication. In those fermentation fields where rapid progress is being made, contributors have laboriously revised and rewritten their chapters several times so that they will be as nearly up-to-date as possible. The editors wish again to express their sincere appreciation to these contributors who have been so generous with their time and effort in this cooperative undertaking.

Over the space of five years, the difficulties of writing, editing, collaborating with distant workers, and producing these volumes covering such a vast and rapidly expanding field introduced possibilities of omissions, errors, repetition, and even minor contradictions. In these matters, the editors ask the indulgence of readers. Despite imperfections, we believe that these volumes are unique in their authoritative treatment of one of the most improtant branches of chemical industry. If this compilation of the current knowledge and industrial practices is found of value by industrial workers and students interested in fermentations, the arduous task has not been completed in vain.

L. A. UNDERKOFLER
R. J. HICKEY

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PART I. THE MICROBIOLOGICAL KETOGENIC PROCESSES

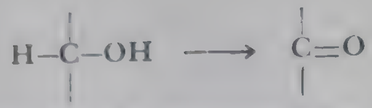
CHAPTER 1

KETOGENIC FERMENTATION PROCESSES

Lewis B. Lockwood

The ketogenic fermentation processess discussed in this chapter are principally those dehydrogenation or oxidation reactions by which polyhydric alcohols, sugars, or polyhydroxy acids are converted respectively into ketoses or keto-sugar acids. Several additional fermentations, including the production of α-keto-glutaric acid and kojic acid will be considered briefly. The most important processes are the oxidation of sorbitol to L-sorbose, and of D-glucose or D-gluconic acid to 2-keto-D-gluconic acid or 5-keto-D-gluconic acid. Numerous other analogous fermentation reactions have been studied on a laboratory scale, but have not been conducted in pilot-plant or factory-size operations. One important fermentation ketone, acetone, is not covered in this chapter on ketogenic fermentations because it is associated with anoxidative processes yielding *n*-butyl alcohol or *n*-butyric acid, and is treated elsewhere (see Chapter 11 of volume I). Acetone is the product of biochemical and physical systems greatly different from those yielding the highly oxidized products discussed here.

The type reaction for the bacterial oxidative ketogenic processes is:



$$H{-}\overset{|}{\underset{|}{C}}{-}OH \longrightarrow \overset{|}{\underset{|}{C}}{=}O$$

The product of the reaction is governed by the choice of substrate and by the specificity of the enzyme system of the fermenting organism. The fundamental requirements of high oxygen tension in the solution and adequate nutrient supply are the same for all the bacterial ketogenic processes considered here.

KETOGENIC PROCESSES OF THE ACETOBACTERS

Many species of the genus *Acetobacter* produce ketogenic dehydrogenations. Butlin[8] has extensively reviewed the biochemical activities of the *Acetobacters.* The first example of this type of biological process was described by Bertrand,[4] who isolated the "sorbose bacterium" from fermenting juice of the berries of the mountain ash (*Sorbus aucuparia L.*) and identified the L-sorbose produced when the sorbitol in the juice was oxidized by the bacteria. Later Bertrand's "sorbose bacterium" was identified with *Bacterium xylinum* Brown, which is now classified as *Acetobacter xylinum.*

Bertrand[5] subjected a considerable number of sugar alcohols and sugars to the action of his organism and, on the basis of his findings, postulated a rule regarding compounds which were and were not oxidized by the organism. Bertrand's rule will be considered in a subsequent section.

The Sorbose Fermentation

The sorbose fermentation is presented first for several reasons. It was the first bacterial ketogenic fermentation discovered, it is one of the simplest of such processes, and it is the process of this type having greatest industrial significance. The operative conditions for all these processes are similar, but some variations, such as the addition of a neutralizing agent or a difference in concentration of substrate may be found in the operation of the different processes.

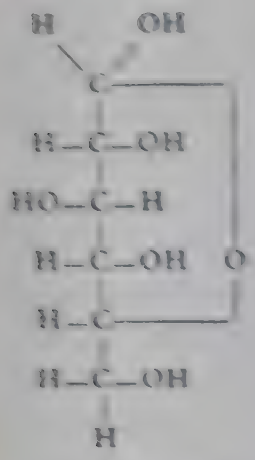
L-Sorbose is produced from the polyhydric alcohol, sorbitol, by the action of several species of bacteria of the genus *Acetobacter. Acetobacter suboxydans* appears to be the species most widely used industrially, although many other species, including *A. xylinum,* *A. gluconicum, A. xylinoides, A. pasteurianum, A. kutzingianum, A. orleanse,* and *A. hoshigaki,* also produce sorbose readily. *A. aceti,* the common vinegar bacterium, does not bring about this fermentation or produces sorbose in yields too poor to be practicable.

As noted previously, the oxidation of sorbitol to sorbose was first described by Bertrand.[4] There was only academic interest in this fermentation until it was discovered that L-sorbose was the logical intermediate in the commercial manufacture of ascorbic acid.

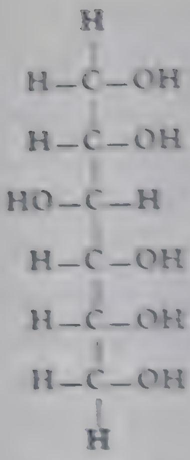
Early studies on the production of sorbose from sorbitol were conducted, using unagitated media, with bacterial pellicles floating on the surface of the solutions. Bertrand's organism, *A. xylinum,* formed very tough, cellulosic pellicles, was slow in its action, and gave relatively poor yields of sorbose. If the pellicles became submerged the fermentation stopped. The fermentation by *A. xylinum* was completed in about 6 weeks and gave sorbose yields of 40 to 60%.

Another organism, *A. suboxydans,* discovered by Kluyver and de Leeuw,[22] formed very light, fragile pellicles, acted much more rapidly than *A. xylinum,* and gave better yields of sorbose. Fulmer, Dunning, Guymon, and Underkofler[14] found that surface fermentations by this organism were complete in about 7 days and gave yields of 80 to 90%. Wells, Stubbs, Lockwood, and Roe[50] grew *A. suboxydans* in vigorously aerated media, under 30 lb gage pressure of air, and obtained rapid oxidation of sorbitol to sorbose. This work was extended by Wells, Lockwood, Stubbs, Roe, Porges, and Gastrock[49] through the semiplant scale. Yields of approximately 98% of theory, based on the sorbitol supplied, were obtained in 14 hours when 10% solutions of sorbitol were fermented. Solutions containing 20% sorbitol required 24 hours and 29% sorbitol solutions required 40 hours.

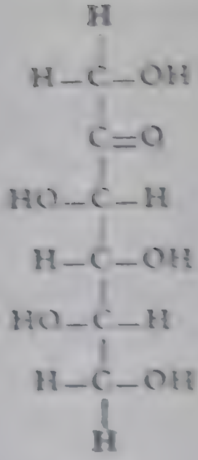
The starting material in the sorbose fermentation is sorbitol (D-glucitol), a polyhydric alcohol, obtained when the aldehydic terminal group of the D-glucose molecule is reduced to a primary alcohol. This reduction is brought about by either electrolytic or catalytic methods. In the first process, some mannitol is also formed. A nickel catalyst is used for the hydrogenation reaction in the catalytic method. It is essential to use sorbitol free of nickel for the fermentation, since *A. suboxydans* is very sensitive to the nickel ion.



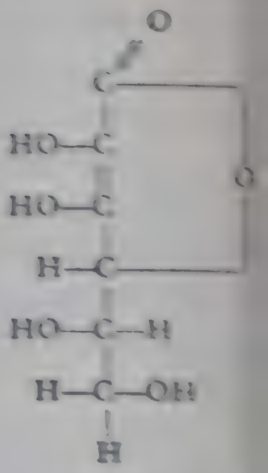
D-Glucose



Sorbitol
(D-Glucitol)



L-Sorbose



L-Ascorbic acid

CULTURE ISOLATION AND CARE

Acetic acid bacteria suitable for the production of sorbose ar
readily isolated from fruits and berries undergoing natural alcoholic
fermentation by yeasts. They are characterized by their ability to
convert ethyl alcohol into acetic acid and to metabolize inorganic
nitrogen compounds, such as ammonium salts. They do not pro-
duce the water-soluble, yellow-green, fluorescent pigments typical
of the genus *Pseudomonas*. Some species of *Acetobacter* require
the addition of vitamins to their culture media. For example
Underkofler, Bantz, and Peterson[44] have found that *p*-aminobenzoi
acid, pantothenic acid, and nicotinic acid are required as growth
factors by *A. suboxydans*, which has been recommended by Land
and Dicken[35] as an assay organism for *p*-aminobenzoic acid. Cul
tures of acetic acid bacteria, including those of *A. suboxydans*, may
be preserved by lyophilization in blood serum or by frequent
transfer on agar slants. The semisolid medium should contain 5%
glycerol, 0.5% yeast extract, and 1.5 to 2% agar. Sorbitol o
glucose may be substituted for the glycerol. However, if glucose i
used in the culture medium, calcium carbonate sufficient t
neutralize the equivalent gluconic acid must be added to main
tain the pH at a suitable value.

Laboratory cultures in liquid medium are prepared for inocu
lation of plant fermentations. Media which contain 5 ml con
steep liquor (50% solids basis) or 5 g yeast extract, or 10 g kille
yeast per liter, in addition to sorbitol, are suitable for the pr
liminary phases of the culture propagation prior to full-size plar
use. The ratio of inoculum for the preparation of successivel

larger cultures should be about 3:100. Each culture should be aerated with ⅓ to 2 volumes of air per volume of culture liquor per minute, the rate depending on the efficiency of dispersion of the air through the medium, the pressure, and the efficiency of agitation. The acetic acid bacteria may be readily overwhelmed in culture if contaminating organisms, such as *Pseudomonas fluorescens, Bacillus cereus, B. subtilis, B. megatherium*, or *Leuconostoc mesenteroides*, are present. Consequently, rigid sanitary precautions are essential in developing the cultures to plant-scale operations. Bacteriophage has not been a problem in *Acetobacter* fermentations and specific bacteriophages for most species of *Acetobacter* are unknown.

PLANT PROCESS

The plant fermentation is conducted in a conventional-type vat fermentor equipped with porous stones, perforated pipes, or other means for the dispersion of air. The fermentor should be constructed of nickel-free metal. High purity aluminum or nickel-free stainless steel is satisfactory for this process. Mechanical agitation may be advantageous, but is not essential, since aeration of the liquor serves also as a means of stirring.

A mash composition suitable for plant-scale fermentation contains:

	per liter	*per gallon*
Sorbitol	100-250 g	0.8-2.0 lb
Corn steep liquor (50% solids)	5 ml	18.9 ml
Antifoam	1 g	3.8 g

Octadecanol, lard oil, or soybean oil are suitable antifoam agents. When the higher sorbitol concentrations are employed, the initial lag period in the fermentation is prolonged. This can be avoided by starting at a 10 or 15% level and feeding additional sorbitol during the logarithmic phase of growth.

The mash is prepared and sterilized by heating under pressure. After cooling, the sterile mash in the fermentor is inoculated with about 3% by volume of active plant culture of *A. suboxydans*, produced in smaller fermentation vessels under aeration. During fermentation, vigorous aeration with sterile air must be provided. Fermentation under an air pressure of 2 atmospheres (30 lb gage pressure) results in a more rapid fermentation, but yields are little different from those obtained when the process is operated at atmospheric pressure. The volume of air supplied will depend on the efficiency with which the solution is oxygenated. The air requirement will be reduced if mechanical agitation and operation under super atmospheric pressure are used. With vigorous stirring at 30 lb gage pressure, $\frac{1}{5}$ volume of air per minute results in rapid fermentation. A higher aeration rate is not beneficial. The preferred temperature range is between 30° and 35°C. Considerable heat is evolved during the fermentation and cooling equipment is necessary in large-scale operations. Coils, jackets, or sprinkler systems have been found satisfactory for temperature control.

ANALYTICAL METHODS

The initial sorbitol content may be conveniently determined by measuring the refractive index of the mash and reading the concentrations from a graph on which the concentration of sorbitol is plotted against refractive index. The progress of the fermentation may be followed by the use of any of the well-known copper-reduction methods for the determination of reducing sugars. A special graph is prepared, on which milligrams of cuprous oxide are plotted against the experimentally determined cuprous oxide-sorbose ratios, or cuprous oxide may be related to sorbose in a table.

RECOVERY OF PRODUCT

Filter aid is added to the fermented liquor and the mash is filtered to remove bacteria and suspended solids. An ordinary filter press is suitable for this step. The clear liquor is concentrated under reduced pressure and crystallized according to the conventional methods for handling sugars. The temperature of evaporation should not exceed 50°C. Analytical yields of 95 to 98% of the sorbitol supplied should be obtained in the fermentation. Actual recovery of 80 to 90% of the sugar should be possible. An activated-carbon treatment is necessary in order to obtain a product of good quality. Wash water from the centrifugation of the crystallized sugar is returned to the filtered beer line.

There are no valuable recoverable by-products of the fermentation. The only residues presenting disposal problems are a small amount of sorbose molasses and the filter cake.

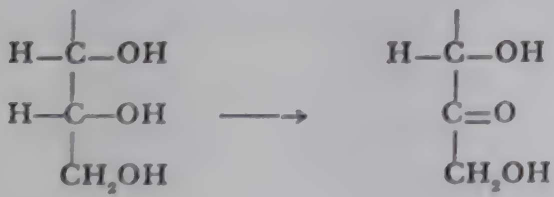
USES, COMPETITIVE PROCESSES, MARKETS

The sole commercial use of sorbose is in the manufacture of ascorbic acid. The nonfermentative steps in the conversion of sorbose to ascorbic acid involve the prepartion of the diacetone derivative which is then oxidized, the acetone groups are removed, and the resultant 2-keto-L-gulonic acid is isomerized to the enediol, with ring closure. Any cheaper method of making ascorbic acid, not involving sorbose as an intermediate, would tend to remove the sorbose fermentation from commercial practice. A process involving the fermentation of L-idonate to 2-keto-L-gulonate by *Pseudomonas mildenbergii* has been patented. This is, however, not competitive at present, since the L-idonate is prepared by hydrogenation of 5-ketogluconic acid, which is itself the product of an *Acetobacter* fermentation.

Production figures for sorbose are not available, but some idea of the quantity produced can be obtained from consideration of the ascorbic acid manufactered. In 1948, 830,528 lb of synthetic ascorbic acid were produced in the United States.

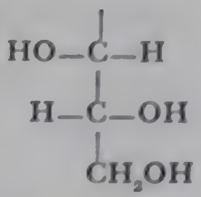
Stereochemical Requirements for Acetobacter Oxidations

The conversion of sorbitol to sorbose is a simple dehydrogenation reaction, typical of those produced by the *Acetobacters* which conform to Bertrand's rule. After the study of the oxidation of numerous alcohols, Bertrand[5] concluded that *Acetobacter xylinum* oxidizes a secondary alcoholic group of a polyhydric alcohol to a ketone when its position is between a primary and a secondary alcohol group and is *cis* to the secondary alcohol:



$$\begin{array}{c} | \\ \mathrm{H-C-OH} \\ | \\ \mathrm{H-C-OH} \\ | \\ \mathrm{CH_2OH} \end{array} \longrightarrow \begin{array}{c} | \\ \mathrm{H-C-OH} \\ | \\ \mathrm{C{=}O} \\ | \\ \mathrm{CH_2OH} \end{array}$$

but

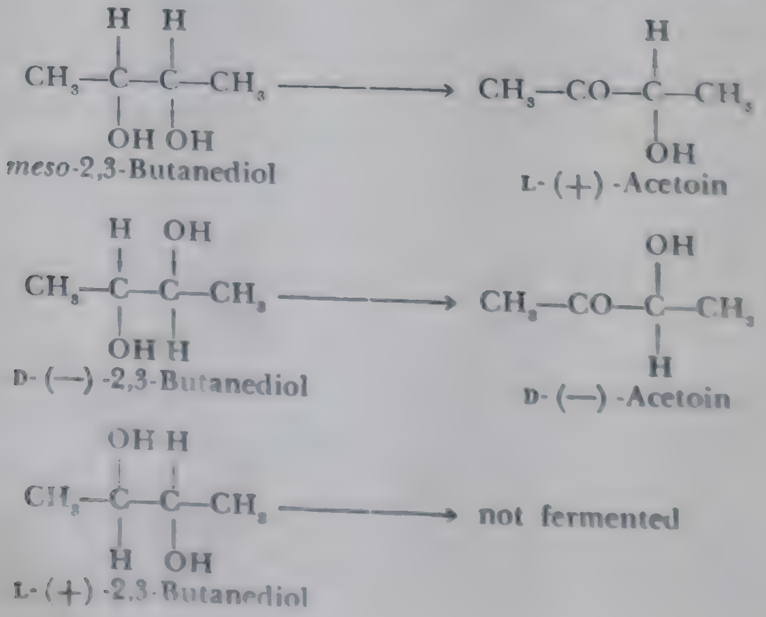


$$\begin{array}{c} | \\ \mathrm{HO-C-H} \\ | \\ \mathrm{H-C-OH} \\ | \\ \mathrm{CH_2OH} \end{array}$$

is not dehydrogenated.

The stereochemical relationship of polyhydric alcohols oxidized by *Acetobacter suboxydans* has been reviewed in considerable detail by Fulmer and Underkofler.[15] They classified the polyhydric alcohols which they studied into four groups, depending on the action of *A. suboxydans* on them: (1) those oxidized in solutions of 25% or greater concentration (sorbitol, mannitol); (2) those not oxidized in solutions at high concentrations, but oxidized readily in solutions of low concentration (glycerol, erythritol); (3) those which require an assimilable substrate for continuous subculture (*meso*-inositol, *meso*-2,3-butanediol, D-(—)-2.3-butanediol); and (4) those alcohols not oxidized even in the presence of an assimilable substrate. The first and second groups are suitable carbon sources for continuous subculture and are oxidized in accordance with Bertrand's rule. The third group can serve as source of energy, but its members are not adequate for the production of cellular substance.

In accordance with Bertrand's rule, mannitol is oxidized to D-fructose, D-perseitol to L-perseulose, D-arabitol to D-xylulose, erythritol to L-erythrulose, and D-gluconates to 5-ketogluconates by *A. suboxydans.* Compounds of a structure not conforming to Bertrand's rule, in which the secondary alcohol is dehydrogenated, include glycerol which yields dihydroxyacetone, 2,3-butanediol which yields acetoin, and *meso*-hexanediol which yields D-hexane-3-one-4-ol. It was shown by van Risseghem[36] that only the D-carbon atom was oxidized when both D- and L-carbons were present in the same molecule, as in *meso*-hexanediol. Whistler and Underkofler[51] demonstrated this with erythritol. Grivsky[17] and Underkofler, Fulmer, Bantz, and Kooi[46] demonstrated the same specificity for D-carbon atoms using isomers of 2,3-butanediol. Here the *meso*-isomer yielded L-(+)-acetoin, the D-(—)-isomer yielded D-(—)- acetoin, but the L-(+)-butanediol which contains no D-carbon atoms was not dehydrogenated.



$$\mathrm{CH_3{-}\underset{OH}{\overset{H}{C}}{-}\underset{OH}{\overset{H}{C}}{-}CH_3 \longrightarrow CH_3{-}CO{-}\underset{OH}{\overset{H}{C}}{-}CH_3}$$

meso-2,3-Butanediol L-(+)-Acetoin

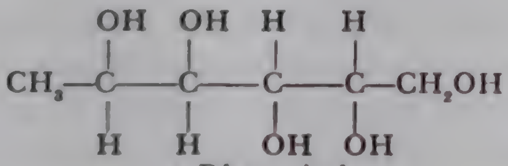
$$\mathrm{CH_3{-}\underset{OH}{\overset{H}{C}}{-}\underset{H}{\overset{OH}{C}}{-}CH_3 \longrightarrow CH_3{-}CO{-}\underset{H}{\overset{OH}{C}}{-}CH_3}$$

D-(—)-2,3-Butanediol D-(—)-Acetoin

$$\mathrm{CH_3{-}\underset{H}{\overset{OH}{C}}{-}\underset{OH}{\overset{H}{C}}{-}CH_3 \longrightarrow \text{not fermented}}$$

L-(+)-2,3-Butanediol

The specificity for D-carbon atoms may explain the failure of the acetic acid bacteria to ferment L-rhamnitol, which contains only L-carbon atoms, but conforms to Bertrand's rule. The enantiomorphic D-rhamnitol is readily fermented.

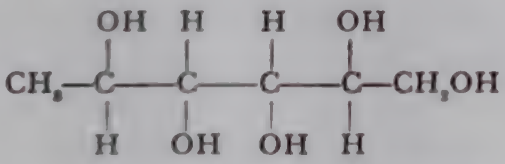


$$\mathrm{CH_3}-\underset{\mathrm{H}}{\overset{\mathrm{OH}}{\mathrm{C}}}-\underset{\mathrm{H}}{\overset{\mathrm{OH}}{\mathrm{C}}}-\underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{C}}}-\underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{C}}}-\mathrm{CH_2OH}$$

L-Rhamnitol

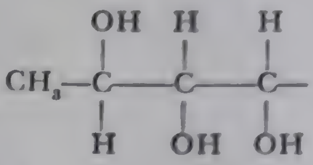
Hann, Tilden, and Hudson,[18] studied the action of *A. suboxydans* on a considerable number of sugar alcohols and extended Bertrand's rule as applied to this organism, stating that the pair of *cis* secondary alcohol groups must have the D-configuration.

One apparently anomalous result of the work of Hann, Tilden, and Hudson was their discovery that L-fucitol



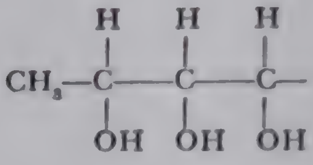
$$\mathrm{CH_3}-\underset{\mathrm{H}}{\overset{\mathrm{OH}}{\mathrm{C}}}-\underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{C}}}-\underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{C}}}-\underset{\mathrm{H}}{\overset{\mathrm{OH}}{\mathrm{C}}}-\mathrm{CH_2OH}$$

was oxidized by *A. suboxydans.* Bollenback and Underkofler[6] confirmed the oxidation of L-fucitol and studied the action of *A. suboxydans* on a number of other ω-desoxy sugar alcohols. They found a number of them, including D-rhamnitol, were oxidized readily by *A. suboxydans* while several others were not attacked. Richtmyer, Stewart, and Hudson[34] identified the oxidation product of L-fucitol as L-fuco-4-ketose. They[35] extended the work of Bollenback and Underkofler to additional ω-desoxy sugar alcohols and found that the configurations



$$\mathrm{CH_3}-\underset{\mathrm{H}}{\overset{\mathrm{OH}}{\mathrm{C}}}-\underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{C}}}-\underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{C}}}-$$

and

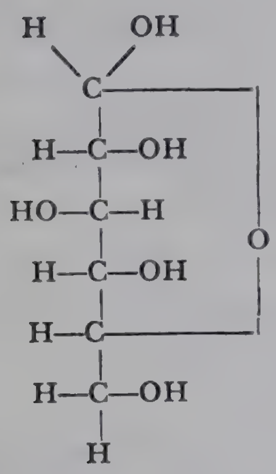


$$\mathrm{CH_3}-\underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{C}}}-\underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{C}}}-\underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{C}}}-$$

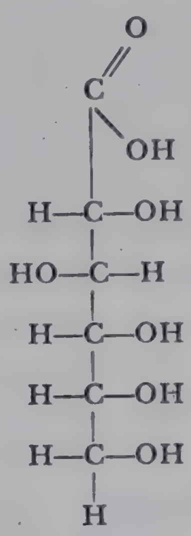
are oxidized by *A. suboxydans,* whereas other types of ω-desoxy sugar alcohols so far tested are not attacked. This permits extension of Bertrand's rule to predict the action of *A. suboxydans* on the ω-desoxy sugar alcohols by considering the $\mathrm{CH_3{\cdot}CHOH}$ group as an elongated $\mathrm{CH_2OH}$ group where one of the hydrogen atoms of the primary alcohol group is replaced by a methyl group.

The 5-Ketogluconic Acid Fermentation

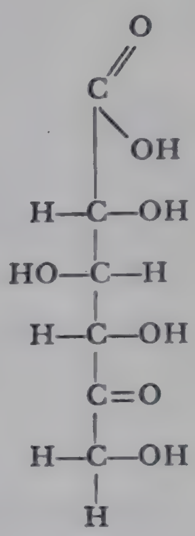
The identification of 5-ketogluconic acid or 5-ketogluconate as a product of the action of acetic acid bacteria was first made by Boutroux[7] in 1880. Hermann and Neuschul[19] later investigated this biochemical reaction. 5-Ketogluconic acid is produced by many species of *Acetobacter* when glucose is fermented, or when calcium gluconate is fermented by special strains. It represents 60 to 90% of the acid formed by various strains of *Acetobacter suboxydans.*



D-Glucose



D-Gluconic acid



5-Keto-D-gluconic acid

Stubbs, Lockwood, Roe, Tabenkin, and Ward[39] studied the production of 5-ketogluconic acid by *A. suboxydans.* They found that gluconic acid is an intermediate in the oxidation of glucose to 5-ketogluconic acid; indeed, no 5-ketogluconic acid is formed until nearly all the glucose in the culture has been oxidized to gluconic acid. All cell multiplication occurs during the period of gluconic acid production, and the further oxidation of gluconic acid to 5-ketogluconic acid is accomplished by already grown nonproliferating cells. The latter oxidation step is accomplished by enzymes already present in the cells, and is much slower than the first step. The initial growth and gluconic acid production step requires 16 to 20 hours to oxidize a 10% glucose solution; and 20 to 40 hours are required for the second step, the over-all time being 35 to 60 hours. Stubbs, Lockwood, Roe, and Ward[40] patented the process for fermentative production of 5-ketogluconic acid. The cultures used in its manufacture are the same as those used for the production of sorbose. The cultural and operating requirements are, consequently, the same, except that it is necessary to add to the medium, at the time of inoculation, 27 g of calcium carbonate for each 100 g of glucose supplied, to neutralize the gluconic acid as it is formed. The calcium carbonate is sterilized dry. The acidity of the glucose solution should be not less than pH 4.5 during sterilization. At slightly alkaline pH values, the Lobry de Bruyn-van Eckenstein reaction occurs, with the consequent rearrangement of a considerable portion of the glucose molecules to a configuration unsuitable for the fermentation.

ANALYTICAL METHODS

Since 5-ketogluconic acid is formed in fermentation liquors only after all the glucose is consumed, an analytical procedure based on the reduction of alkaline copper sulfate can be used to follow the entire fermentation. Glucose is determined by any of the conventional sugar methods during the first phase of the fermentation. The slight solubility of calcium 5-ketogluconate, about 0.25%, makes necessary considerable care in sampling during the later phase of the fermentation. The mash must be well agitated during sampling and a measured volume of sample taken. A measured quantity of concentrated hydrochloric acid is added to the entire sample and the final volume recorded after solution of the calcium 5-ketogluconate is complete. The 5-ketogluconic acid in the acidified sample is determined by the method of Shaffer and Hartmann,[38] using previously determined cuprous oxide-ketogluconic acid ratios. A final correction is made for the dilution of the sample on acidification.

RECOVERY AND PURIFICATION

Calcium 5-ketogluconate precipitates during the later stages of the fermentation, and is filtered off at harvest. The crude substance is dissolved in dilute hydrochloric acid, treated with activated carbon, and refiltered with filter aid to remove bacteria. the calcium 5-ketogluconate is reprecipitated in the filtrate by slow addition of ammonia while stirring rapidly. The recrystallized salt is filtered and washed free of ammonium chloride with water. The yield of 5-ketogluconic acid should be 90 to 95 g from 100 g of glucose. A very little 2-ketogluconic acid is also formed in this process, and remains in the original filtrate.

USES AND COMPETITIVE PRODUCTS

5-Ketogluconic acid may be used as an intermediate in the production of D-tartaric acid. It is oxidized to this acid by air, in the presence of a vanadium pentoxide catalyst.[1] The economy of the fermentation depends on the demand for tartaric acid, which is a by-product of the wine industry. Recent improvements in the processing of wines, lees, and argols would indicate that tartaric acid from the wine industry will continue to be cheaper than the acid made from 5-ketogluconic acid. An additional process for the production of tartaric acid by the oxidation of starch is also competitive.

Other Acetobacter Fermentations of Practical Importance

Although the production of sorbose, and possibly of 5-ketogluconic acid, are the only large-scale *Acetobacter* fermentations of industrial importance, the action of *Acetobacter suboxydans* on sugar alcohols of suitable structure, conforming to Bertrand's rule, furnishes a convenient method for obtaining certain otherwise unobtainable ketose sugars. Several of these sugars have been produced by laboratory investigators by this method for different purposes. For example, Whistler and Underkofler[51] produced L-erythrulose from erythritol, Tilden[43] obtained L-perseulose from D-perseitol, and Underkofler and Fulmer[45] developed an efficient method for producing dihydroxyacetone from glycerol. Dihydroxyacetone has considerable importance as a fine chemical and is produced commercially in large-scale laboratory apparatus by this fermentation process to supply the limited market. The *Acetobacters* oxidize glucose to gluconic acid, galactose to galactonic acid, etc., and selected strains may be utilized for these fermentations (Currie and Carter;[12] Takahashi;[41] Takahashi and and Asai[42]). However, mold fermentations or chemical oxidation of glucose supply the market for gluconic acid and calcium gluconate. These processes have advantages over the *Acetobacter* fermentation for producing gluconic acid, since this compound is further converted to ketogluconic acids by most *Acetobacter* species (see Chapter 14 of volume I).

With the strains of *A. suboxydans* employed by investigators in the United States, the principal oxidation product obtained by action of this organism on glucose in the presence of calcium carbonate or on calcium gluconate has been identified as calcium 5-ketogluconate. However, there is apparently considerable strain difference with various *Acetobacters.* Bernhauer and Görlich[3] identified 2-ketogluconate formed, along with much larger amounts of 5-ketogluconate, by *A. gluconicum.* Recently Riedl-Tumova and Bernhauer[33a] reported that a strain identified as *A. suboxydans muciparum* produced mainly 5-ketogluconate, a strain of *A. suboxydans* produced principally 2-ketogluconate, while *A. melanogenum* produced another as yet unidentified ketogluconic acid salt along with 5-ketogluconate.

Inositol is oxidized to keto-inositol by *Acetobacter suboxydans.* Dunning, Fulmer, Guymon, and Underkofler[13] first prepared a keto-inositol by oxidation of *meso*-inositol with *A. suboxydans.* Kluyver and Boezaardt[20] obtained a mono-keto-inositol identical with the inosose of Posternak.[33] Carter, Belinskey, Clark, Flynn, Lytle, McCasland, and Robbins[11] also obtained inosose in good yields by the oxidation of *meso*-inositol by *A. suboxydans.* Magasanik and Chargaff[28] studied the oxidation of various inositols by *A. suboxydans* and found that *meso*-inositol gave *meso*-inosose (monoketo), *epi*-inositol gave *l-epi*-inosose and probably *d-epi*-inosose (monoketo), while *d*- and *l*-inositol gave α-diketoinositols.

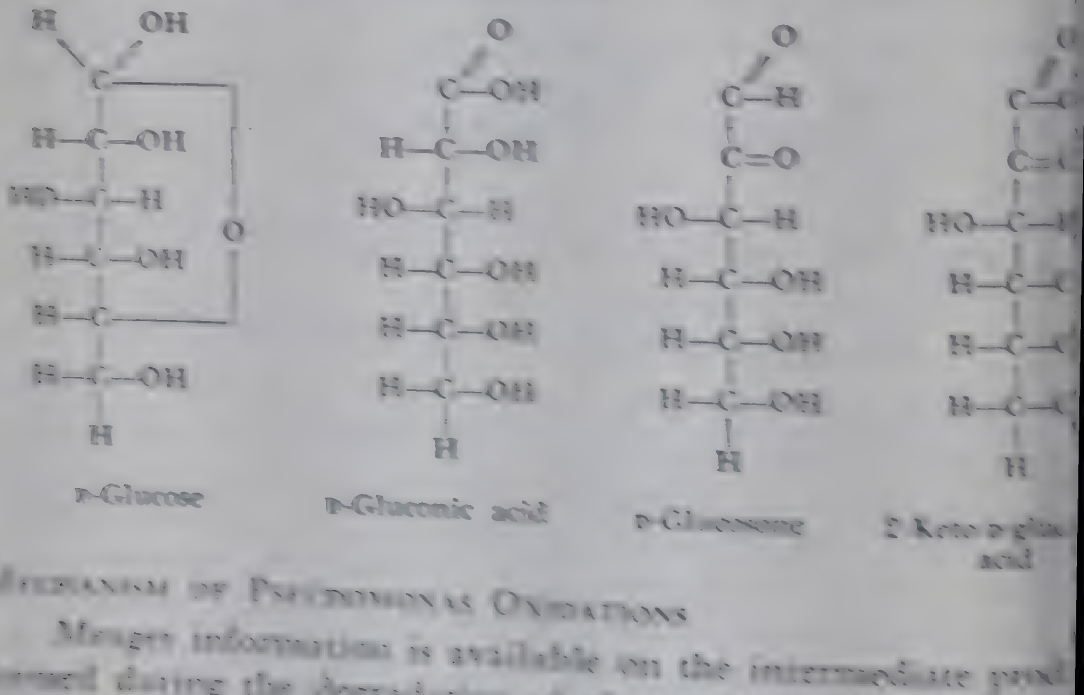
KETOGENIC PROCESSES OF THE PSEUDOMONAS

The 2-Ketogluconic Acid Fermentation

2-Ketogluconic acid has been recognized as a keto acid produced by *Acetobacter* species. Bernhauer and Görlich[3] were apparently the first workers to recognize this acid in the fermentation products from *A. gluconicum.* However, the 2-ketogluconic acid is produced in relatively minor amounts by most *Acetobacters,* and along with the 5-ketogluconic acid. It may represent as much as 10 to 40% of the total final acid yield in 5-ketogluconic acid fermentations. The actual ratio of 2-ketogluconic acid to 5-ketogluconic acid depends on the strain of *Acetobacter* chosen.

Practical means for the production of 2-ketogluconic acid were first presented by Stubbs, Lockwood, Roe, Tabenkin, and Ward[39] who described the production of this acid in submerged cultures by an organism isolated from aberrant 5-ketogluconic acid fermentation liquors. The culture was later identified by Lockwood, Tabenkin, and Ward[26] as a strain of *Pseudomonas fluorescens* and a survey of other species revealed that the property of oxidizing glucose to 2-ketogluconic acid was widespread in the genus P *domonas*. The acid is produced by *P. aeruginosa, P. fragi, P. mil bergii, P. ovalis, P. pavonacea, P. putida,* and *P. schuylkilliensi*

2-Ketogluconic acid is produced by *Pseudomonas* species w glucose or gluconate is the substratum in vigorously aerated cultu Gluconic acid is an intermediate in the oxidation of glucose 2-ketogluconic acid. 2-Ketogluconic acid is structurally relate both gluconic acid and glucosone and may be derived from ei by chemical oxidation. Glucosone is not known as a fermenta product and the ability of bacteria to oxidize glucosone to ketogluconic acid has not been reported. The enzyme system sponsible for the *Pseudomonas* ketonization differs from that of Bertrand oxidation by *Acetobacter* in that one of a *trans* pai secondary alcoholic groups is oxidized, while the Bertrand enz system requires the *cis* configuration.



D-Glucose D-Gluconic acid D-Glucosone 2-Keto-D-gluc acid

MECHANISM OF PSEUDOMONAS OXIDATIONS

Meager information is available on the intermediate prod formed during the degradation of glucose by oxidative organ such as *Pseudomonas* species. These organisms are obligate aer and without intense aeration, the end products of glucose meta ism are carbon dioxide and water. Under conditions of vig aeration, Lockwood, Tabenkin, and Ward[19] found a numbe cultures of *Phytomonas* and *Pseudomonas* which yielded 58 to gluconic acid or better than 70% 2-ketogluconic acid from glu With intense aeration, these acids appear as the end product the oxidation of glucose by *Pseudomonas* species.

Recently, workers at the University of British Columbia have een publishing a series of papers on their investigations of the ntermediate metabolism of *Pseudomonas aeruginosa.* This organ-sm has been shown to produce appreciable quantities of 2-keto-luconic acid from glucose under conditions of intense aeration,[33] ut in still cultures, it metabolizes glucose to carbon dioxide and ater. By paper chromatography, Norris and Campbell[24] showed hat both gluconic and 2-ketogluconic acids were present in de-ectable amounts in 16- and 24-hour cultures of *P. aeruginosa* rown in still culture in glucose-ammonium phosphate liquid nedium. After 38 hours, all glucose had disappeared and only the aintest trace of gluconic or 2-ketogluconic acids could be detected hromatographically. Gluconic and 2-ketogluconic acids as sub-trates were found to be oxidized rapidly by *P. aeruginosa.* It was oncluded that gluconic and 2-ketogluconic acids were being formed t a continuous rate and being removed by continuous oxidation nd that, therefore, these compounds were part of the system hrough which most if not all of the glucose was oxidized. Glu-onic and 2-ketogluconic acids are evidently intermediates in the xidation of glucose by *P. aeruginosa.*

Campbell and Norris[9] showed that neither the fermentation iquor nor the cells of the organism contained any of the hexose hospate members of the Embden-Meyerhof scheme of glucose issimilation. Resting cells of the organism failed to remove any lucose or fructose or to take up phosphate. It was concluded that *. aeruginosa* did not initiate dissimilation of glucose by the mbden-Meyerhof pathway. Rather, under normal physiological onditions, the organism oxidizes glucose by a pathway not pre-iously reported in any type of tissue or bacterial cell, through ormation of gluconic and 2-ketogluconic acids as intermediate roducts.

Further work by Warburton, Eagles, and Campbell[47] identified yruvate as a later intermediate in the oxidation of glucose by *P. eruginosa* and Campbell and Stokes[10] detected the presence of tricarboxylic acid cycle in the metabolism of this organism. Much rther work will be necessary to fully elucidate the complete echanism of the oxidative dissimilation of glucose by *P. aerugi-osa.* It will be of interest to ascertain whether a similar pathway rough gluconic acid may be employed by other oxidative ganisms, such as some of the *Acetobacters* and fungi.

CULTURES AND CULTURE CARE

The *Pseudomonas* organisms responsible for the oxidation of glucose to 2-ketogluconic acid are extremely widespread and can be readily isolated from soil. In fact, they are among the most common soil organisms. Suitable strains occur in temperate climates in almost all fertile soils except those which are very acid. Some of the *Pseudomonas* cultures which give good yields of 2-ketogluconic acid are plant or animal pathogens, but suitable nonpathogenic strains are also easily isolated.

Cultures may be stored in dry loam according to the method of Greene and Fred,[16] or as slants on vegetable or liver decoction agar media.

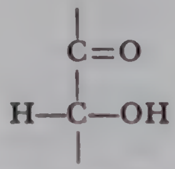
THE FERMENTATION OF GLUCOSE TO 2-KETOGLUCONIC ACID

2-Ketogluconic acid has not been manufactured on a commercial scale. However, equipment used for the manufacture of sorbose should be entirely suitable for the production of this acid. Rigid exclusion of nickel is not necessary, since *Pseudomonas* are remarkably insensitive to this metallic ion. The temperature, aeration, and agitation optima are the same for the three fermentations: sorbose, 5-ketogluconic acid, and 2-ketogluconic acid. In addition to the 100 g of glucose, 5 ml of corn steep liquor, and 27 g of calcium carbonate per liter required in the 5-ketogluconate fermentation, it is necessary to supply 0.25 g of $MgSO_4{\cdot}7H_20$, 0.6 g of KH_2PO_4, and 2 g of urea per liter. The urea must be sterilized separately from the glucose; this is usually done by autoclaving a 20% solution. The calcium carbonate is sterilized dry, and added at the time of inoculation. A sterile, aqueous slurry could also be used. The composition of the media for the preliminary propagative steps and the final fermentation is the same. The ratio of inoculum to inoculated mash is about 3:100. The fermentation is conducted under 30 lb gage pressure, at 30°C. It is complete in 32 to 35 hours.

Contamination has not been a serious problem in pilot-plant studies. Several bacteriophages for *Pseudomonas aeruginosa* are known. Consequently, it appears that bacteriophages may occur and cause trouble in industrial operation of this process. Immunization of cultures may become necessary.

ANALYTICAL METHODS

2-Ketogluconic acid is produced in culture media while a considerable quantity of glucose remains in the mash. The analysis of these liquors is ordinarily based on the reduction of alkaline copper sulfate, as in the determination of glucose, sorbose, 5-ketogluconic acid, or other compounds having the configurational group:



$$\begin{array}{c} | \\ \mathrm{C{=}O} \\ | \\ \mathrm{H{-}C{-}OH} \\ | \end{array}$$

Cuprous oxide : 2-ketogluconic acid ratios have been determined and may be used in either tabular or graphic form.

Since both glucose and 2-ketogluconic acid reduce alkaline copper reagents, such as Benedict's solution, it is necessary to differentiate between the copper reduction of each of these two compounds. This is done on the basis of optical rotation. The rotation of 1% glucose solution is +0.55°, whereas that of a 1% solution of 2-ketogluconic acid is −0.88°. An algebraic equation has been worked out to facilitate the calculation of the 2-ketogluconic acid and glucose contents of such mixtures:

$$CuK = 0.353CU - 13.28\alpha$$

CuK is the cuprous oxide produced by the reducing action of 2-ketogluconic acid on cupric ions; CU is the total cuprous oxide, and α is the observed rotation of the sample in a 1 dm tube. The total cuprous oxide less the cuprous oxide due to the 2-ketogluconic acid equals the cuprous oxide, CuG, produced by reduction of cupric ions by glucose:

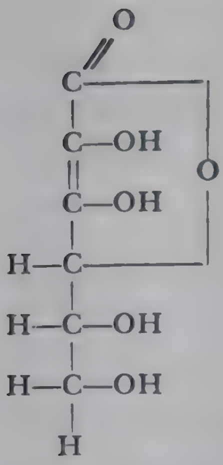
$$CU - CuK = CuG$$

These cuprous oxide values may be readily converted to glucose or 2-ketogluconic acid by use of appropriate tables or graphs.

RECOVERY AND USES OF 2-KETOGLUCONIC ACID

The 2-ketogluconic acid is recovered as the calcium salt on crystallization from the filtered, evaporated culture liquor. The concentration is done under reduced pressure, so that the temperature does not exceed 50°C. No by-products of this fermentation are to be expected, since yields as great as 90% of theory can be obtained. Some calcium salt of gluconic acid, or of acids which form on further oxidation of 2-ketogluconic acid, may be present.

The principal use for 2-ketogluconic acid appears to be as an intermediate in the preparation of D-araboascorbic acid.



D-Araboascorbic acid

This compound has much less vitamin C potency than does L-ascorbic acid, but exerts a vitamin C sparing antioxidant action in preserving fruits and other food products. Some fatty acid esters of D-araboascorbic acid may find use as antioxidants in the prevention of rancidity in oils and fats of vegetable or animal origin. The leading competitor of D-araboascorbic acid will undoubtedly be L-ascorbic acid.

∝-Ketoglutaric Acid Fermentation

Lockwood and Stodola[24] found that ∝-ketoglutaric acid, $HOOC{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}COOH$, is produced when a 2-ketogluconic acid fermentation by certain strains of *Pseudomonas fluorescens* is allowed to proceed beyond the point of maximum 2-ketogluconic acid content. At this time, the aeration rate is reduced to approximately half the initial rate. At the point at which all the 2-ketogluconic acid has been destroyed, about 25 g of ∝-ketoglutaric acid is present for each 100 g of glucose initially supplied. A patent on this process has been granted to Lockwood and Stodola.[25]

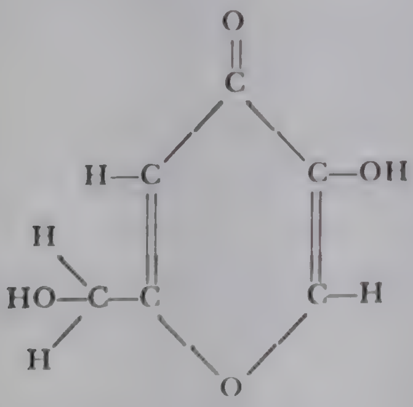
More recently, Koepsell, Stodola, and Sharpe[19a] have reported yields of about 41 g of ∝-ketoglutaric acid per 100 g of glucose supplied in shake-flask cultures. They employed the same organism, *Ps. fluorescens* NRRL B-6, and a medium containing, per 100 ml, 9 g glucose, 0.32 g $(NH_4)_2SO_4$ (urea, sterilized separately, could be substituted on an equimolar basis), 0.11 g KH_2PO_4, 0.024 g $MgSO_4$, 1 ppm Fe supplied as ferrous ammonium sulfate, and 3.75 g $CaCO_3$ (sterilized separately). In the fermentations, conversion of glucose to gluconate and 2-ketogluconate had occurred at 40 hours, but appreciable α-ketoglutarate was not detectable until after 60 hours. About 11 days were required to achieve maximum yields of α-ketoglutarate. In many cases, calcium α-ketoglutarate separated during fermentation from the medium, which became a thick slurry by the end of fermentation. Sometimes supersaturation occurred and seeding was necessary to start precipitation. For recovery of the free acid, the whole culture was acidified with hydrochloric acid and extracted with ethyl acetate. Crude crystalline α-ketoglutaric acid was obtained by concentrating the extract and the pure acid by recrystallization from ethyl acetate–petroleum ether. The authors stated that the ease of recovery would enhance the possibility of producing the substance on an industrial scale by fermentation methods.

Pseudomonas Oxidation of Inositol

Kluyver, Hof, and Boeezaardt[21] have reported that inositol is oxidized by *Pseudomonas beijerinckii* to a triketo-inositol which oxidizes to tetrahydroxybenzoquinone.

KOJIC ACID FERMENTATION

Kojic acid was first identified as a mold metabolic product and its structure was worked out by Yabuta.[52] Saito[37] had previously observed the compound when ferric chloride was added to the culture filtrates of *Aspergillus oryzae.* It is a pyrone compound, the acidic properties of which are due to the enolic hydroxyl group. No carboxyl group is present.



Kojic acid

Pioneering work on the production of kojic acid by mold fermentation was reported by May, Moyer, Wells, and Herrick.[29]

The acid is produced by members of the *Aspergillus flavus-oryzae* and *A. tamarii* groups, growing on the surface of the culture solution. There is considerable variation from strain to strain in the ability to produce kojic acid.

A suitable nutrient composition for the production of kojic acid contains 200 g glucose, 0.054 g H_3PO_4, 0.1 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, and 1.125 g NH_4NO_3 per l. The addition of ethylene chlorohydrin, up to 0.1 g per l of culture solution, appears to result in considerable increase in yield.[30]

Kojic acid is synthesized by the mold from glucose, sucrose, xylose, or dihydroxyacetone. Consequently, it appears probable that the larger sugar units are broken down into smaller fragments, from which the acid is synthesized.

Barham and Smits[2] reported an extensive investigation of conditions required for the maximum production of kojic acid from xylose by surface cultures of *A. flavus.*

Since kojic acid contains a carbonyl group adjacent to a secondary alcoholic group, the compound reduces alkaline copper reagents, such as Fehling's or Benedict's solution. The kojic acid reduction values published by May, Moyer, Wells, and Herrick[29] are:

Kojic acid, mg	5	10	20	30
Cuprous oxide, mg	9.5	17	35	52

Correction for copper reduced by kojic acid is made in the determination of sugar in kojic acid culture liquors by copper reduction methods. The amount of correction to be applied depends on the quantity of kojic acid present. This is determined by titration using alizarin orange as indicator.

The yield of kojic acid should be about 45 g per 100 g of glucose supplied, after a 12-day fermentation period. The produc crystallizes out readily on evaporation of the culture liquor. T obtain a colorless product, iron must be carefully excluded, sinc minute traces of this metal will give a deep red color with koji acid.

Although kojic acid is easily produced by fermentation, n uses for the compound have been developed. Should uses be found undoubtedly submerged processes would be developed to replac the surface process.

OTHER KETOGENIC FERMENTATION PROCESSES

Several ketogenic processes besides those previously mentioned have been described, in addition to the many *Acetobacter* oxidations in accordance with Bertrand's rule. Among these should be mentioned the production of acetoin by yeast and by *Bacillus polymyxa, Aerobacter aerogenes, A. cloacae,* and various species of *Proteus* (see Chapter 2). The metabolism of these bacteria is essentially of the butanediol-producing type, but the diol may be oxidized to acetoin if the cultures are aerated. Bacteria of the genus *Pseudomonas* have been reported to oxidize the hydroxyl group on the side chain of a steroid associated with adrenal cortical hormone to the corresponding ketone, thus increasing the sex-hormonal potency of the extracts of the adrenal cortex.

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PART II. THE FERMENTATIVE PRODUCTION OF 2,3-BUTANEDIOL

HAPTER 2

FERMENTATIVE PRODUCTION OF 2,3-BUTANEDIOL

G. A. Ledingham and A. C. Neish

INTRODUCTION

Interest in four-carbon compounds as possible precursors for
he manufacture of 1,3-butadiene during World War II led to
nany studies on the production of 2,3-butanediol, also called 2,3-
utylene glycol, by government, university, and industrial labora-
ories in the United States and Canada. As a result of these studies,
nowledge available early in the century on the production of this
ompound by different becteria has been greatly expanded and sev-
ral new fermentations have been described. None of these processes
as actually been applied commercially, but large-scale pilot-plant
perations have been conducted. Although not utilized industrially
t present, an extensive treatment of the development work con-
ected with the 2,3-butanediol fermentation is justified, not only be-
ause of its commercial potential, but also to illustrate the progress
hich can result from intensive research on a fermentation process.
ince the work of the various laboratories was correlated through
onferences and free exchange of information, there has been little

hesitancy in publishing results. Consequently, the literature relating to this fermentation is voluminous. Some five hundred references, more or less closely connected with the microbiology, production, and analysis of 2,3-butanediol and related compounds, are in the files of the authors. Even by limiting references to those directly relating to the discussion, the bibliography at the end of this chapter has become quite lengthy.

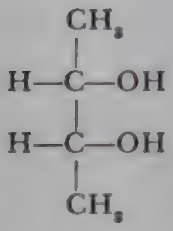
One of the earliest references to the natural occurrence of butanediol is possibly that of Henninger,[91] who isolated what he thought was isobutylene glycol from wine in 1882. Later it was found that pure-yeast fermentations produced the same compound.[41] It is now known that yeast produces small quantities of 2,3-butanediol and it is very likely that this compound was isolated by Henninger. The first oxidation product of 2,3-butanediol, acetylmethylcarbinol or acetoin, has also been recognized as a product of bacterial fermentations since 1901.[79] In 1906, Harden and Walpole[89] first proved that bacteria produced 2,3-butanediol in appreciable quantities, using cultures which would now be classified as *Aerobacter aerogenes*. Walpole[263] reported further studies in 1911 and Scheffer[216] in 1928. Donker[61] published the first important contribution to the *Bacillus polymyxa* fermentation in 1926. The initial work of Donker and Scheffer led to a number of patents typical of which were those of Verhave,[259,260,261] Kluyver and Scheffer,[110,111,112] and Scheffer.[217] In 1933, Fulmer, Christensen, and Kendall,[73] at Iowa State College, showed that the *Aerobacter aerogenes* fermentation had industrial possibilities, because they were able to obtain good yields in much shorter time than previously. Many other contributions were later made by Werkman, Fulmer, Underkofler, and their students from this College.

The wartime investigations on the production of 2,3-butanediol were begun early in 1942 in both the United States and Canada. The Northern Regional Research Laboratory of the United States Department of Agriculture in Peoria, Ill., held a number of conferences which were attended by representatives from Iowa State College, University of Wisconsin, University of Alberta, Commercial Solvents Corporation, Schenley Distillers, Joseph E. Seagram and Sons, Inc., Lucidol Corporation, Heyden Chemical Corporation, Merck and Company, Columbia Brewing Company, and the National Research Council of Canada. Progress on the problem was greatly expedited through the pooling of reports and exchange of cultures. Laboratory studies were followed by pilot-plant investigations in the Northern Regional Research Laboratory, Joseph E. Seagram and Sons, Inc., Louisville, Ky., Schenley Distillers, Lawrenceburg, Ind., Commercial Solvents Corporation, Terre Haute, Ind., and the National Research Laboratories, Ottawa, Canada. Most of this wartime work was concentrated on the *Aerobacter* and the *Bacillus polymyxa* fermentations and on efforts to convert the 2,3-butanediol to 1,3-butadiene.

PROPERTIES OF 2,3-BUTANEDIOL IN RELATION TO POSSIBLE USES

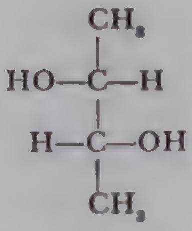
Physical Properties

2,3-Butanediol is a colorless, odorless, viscous liquid. It exists in three stereoisomeric forms, which are depicted by the conventional planar projection formulas as:



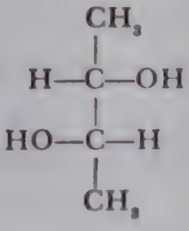
$$\begin{array}{ccc} & CH_3 & \\ & | & \\ H— & C & —OH \\ & | & \\ H— & C & —OH \\ & | & \\ & CH_3 & \end{array}$$

meso-2,3-Butanediol



$$\begin{array}{ccc} & CH_3 & \\ & | & \\ HO— & C & —H \\ & | & \\ H— & C & —OH \\ & | & \\ & CH_3 & \end{array}$$

D-(—)-2,3-Butanediol



$$\begin{array}{ccc} & CH_3 & \\ & | & \\ H— & C & —OH \\ & | & \\ HO— & C & —H \\ & | & \\ & CH_3 & \end{array}$$

L-(+)-2,3-Butanediol

The D and L forms are mirror images and thus are identical in all common physical properties except the direction of rotation of polarized light. All three stereoisomers are found in nature in varying proportions, depending on the organism which makes them. This is illustrated in Table 1. Most bacteria give predominantly *meso*-2,3-butanediol, but the dextro-rotatory and levo-rotatory isomers are also found, particularly the second which is formed to the practical exclusion of all the other isomers by *Bacillus polymyxa.* The values given in Table 1 for the composition of the diol mixture are rather approximate. *A. aerogenes* is reputed to give a small amount of *racemic*-2,3-butanediol[26,249] and some of the other species may produce this also.

TABLE 1. OPTICAL ROTATION OF 2,3-BUTANEDIOL[a] PRODUCED BY VARIOUS BACTERIA

Organism	$[\alpha]_D^{23-26°C}$	Approximate Composition of mixture	References
Aerobacter aerogenes	+0.8 to + 1.8	5-14% L-(+), remainder *meso*	26, 70, 89, 152, 263
Bacillus polymyxa	—13.0 to —13.34	At least 98% D-(—)	115, 132, 156 209, 265
Pseudomonas hydrophila	—1.25	About 8% D-(—), remainder *meso*	43, 231
Bacillus subtilis (Ford's type)	—3.86 to —5.0	Up to 40% D-(—), remainder *meso*	21,166
Serratia marcescens	+0.07 to +0.35	Predominantly *meso*	167
Serratia plymuthicum	—0.18	Predominantly *meso*	168
Serratia anolium	+0.03	Predominantly *meso*	168

[a] Diol samples purified by distillation before measurement of the optical rotation.

Some of the physical properties of the stereoisomers of 2,3-butanediol are summarized in Table 2. The pure dextro-rotatory isomer has not been obtained, but its properties should correspond to those of the levo-rotatory isomer except for the sign of rotation. *Racemic*-2,3-butanediol acts as a fourth compound with somewhat different physical properties from those of the pure isomers and its identity as an equimolecular mixture of the D and L forms can be shown only by resolving it with optically active agents. It has been shown by biochemical[80] and chemical[152] studies that

TABLE 2. PHYSICAL PROPERTIES OF THE STEREOISOMERIC 2,3-BUTANEDIOLS

Constant	*Meso*-2,3-butanediol	D-(—)-2,3-butanediol	*Racemic*-2,3-butanediol	References
Melting point, °C	34.4	19.0	7.6	265, 279
Boiling point, °C at 745 mm	181-182	179-180	177	132, 152, 279
Specific rotation at 26°C (D line)	0.00°	—13.34°	0.00°	156
Density, g per ml at 25°C	0.9939	0.9869	—	115
Refractive index at 25°C	1.4366	1.4308	—	42, 115
Surface tension, dynes per cm at 25°C	—	30.61	—	115
Viscosity, centipoises at 35°C	65.6	21.8	—	115
Specific viscosity at 30°C	15.72	5.34	—	132
Melting point of di-*p*-nitrobenzoate, °C	193	143	128	209
Specific rotation of di-*p*-nitrobenzoate in chloroform at 25°C	—	51.0°	—	209

2,3-butanediol and acetoin have the configuration D-(—) and L-(+). In the balance of this chapter, the symbols (—) and (+) will be used for levo- and dextro-rotatory 2,3-butanediols, respectively.

It is obviously of practial importance to determine the ratio of the isomers in the 2,3-butanediol produced by any given organism. There are many strains of bacteria for which this has not been done. Methods have been suggested based on measurements of the specific viscosity, or the specific conductivity, or pH in boric acid solutions.[132] Some use might be made of the observation that the rotation of 2,3-butanediol is six hundred times as great in cuprammonium solution as in water.[202] A quantitative method might be based on specific oxidation of 2,3-butanediol isomers by different bacteria.[233] It is necessary to interpret with caution the results of physical measurements on purified samples, since 2,3-butanediol is extremely hygroscopic[132] and a small amount of water produces a relatively large change in optical rotation and some other properties.[42]

It is evident from the data of Table 2 that *meso*-2,3-butanediol has quite different physical properties from those of the other isomers. In addition, differences have been noted in the Raman spectra.[245]

One of the greatest differences, which is of considerable practical importance, is in the freezing point–composition diagrams of 2,3-butanediol-water mixtures (see Figure 1). Both (—) and *meso* isomers form hydrates as shown by the effect of water on the density and specific rotation, but the hydrate formed by the *meso* isomer crystallizes readily while that formed by the (—) isomer does not. As a result of this difference the *meso* isomer is useless as an antifreeze while careful studies of the (—) isomer in binary [43] and ternary[41] mixtures have shown that it may be considered as a substitute for ethylene glycol in permanent type antifreezes. This use has been patented in both Canada[44] and the United States.[46]

In addition to its use as an antifreeze, 2,3-butanediol might find other applications based on its low vapor pressure and hygroscopicity. One that has been suggested[113] is for the moistening and softening of glue, gelatin, casein, tobacco, composition cork, textile fibers, etc. The *meso* isomer would probably be as good as the (—) isomer for these uses.

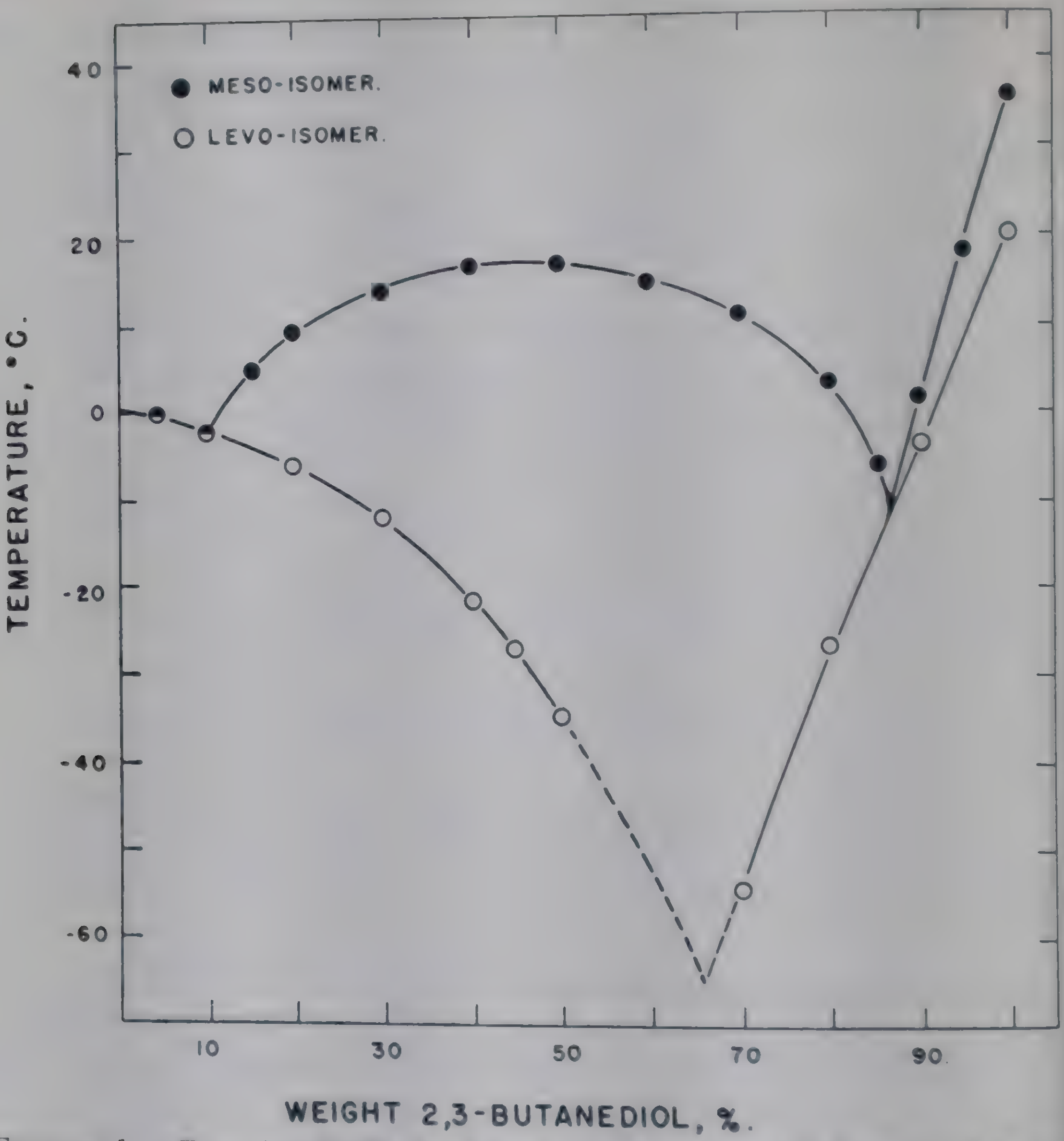
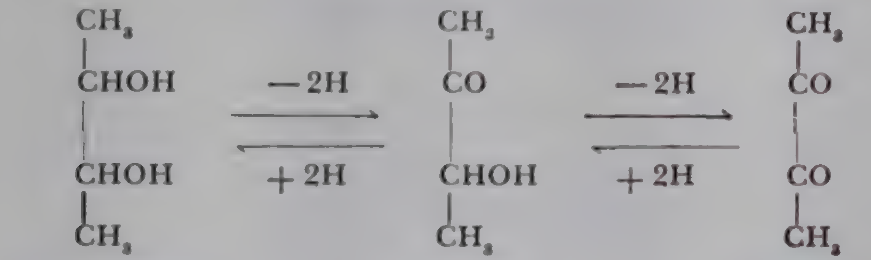


FIGURE 1. *Freezing-Point Diagram of Aqueous Solutions of* Levo- *and* Meso-*2,3-Butanediols*

Information is available on the vapor pressure of 2,3-butanediol and detailed studies have been made of vapor-liquid equilibria of the system 2,3-butanediol-water for both of these isomers.[24,115,189,218,254] Measurements have been made of ultrasonic velocity in 2,3-butanediol[276,277] and also of its heat of formation.[15

Chemical Properties

Certain potential uses of 2,3-butanediol are based on its conversion to other chemicals. Some of the products which can be formed in good yields by processes which might be commercially feasible are shown in Table 3. Space here permits brief discussion of only a few of these reactions. Acetoin and diacetyl are formed by oxidation of 2,3-butanediol:

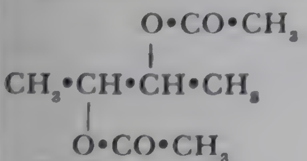


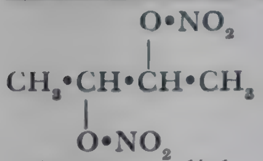
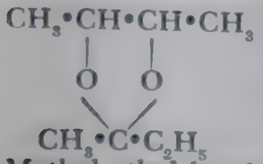
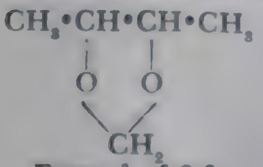
$$CH_3{\cdot}CHOH{\cdot}CHOH{\cdot}CH_3 \underset{+2H}{\overset{-2H}{\rightleftharpoons}} CH_3{\cdot}CO{\cdot}CHOH{\cdot}CH_3 \underset{+2H}{\overset{-2H}{\rightleftharpoons}} CH_3{\cdot}CO{\cdot}CO{\cdot}CH_3$$

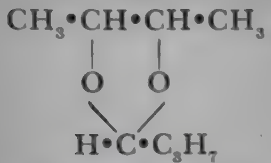
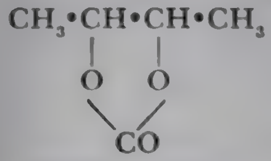
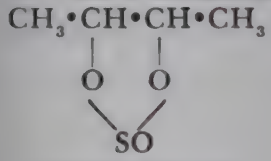
2,3-Butanediol Acetoin Diacetyl

This reaction can be carried out by bacteria in the presence of air and it is possible to build up appreciable concentrations of acetoin in the medium. Thus acetoin can be produced directly by fermentation of carbohydrates under aerobic conditions. Alternatively, 2,3-butanediol can be converted to acetoin and diacetyl by catalytic oxidation of dehydrogenation over copper catalysts.[23,53,116,145,157]

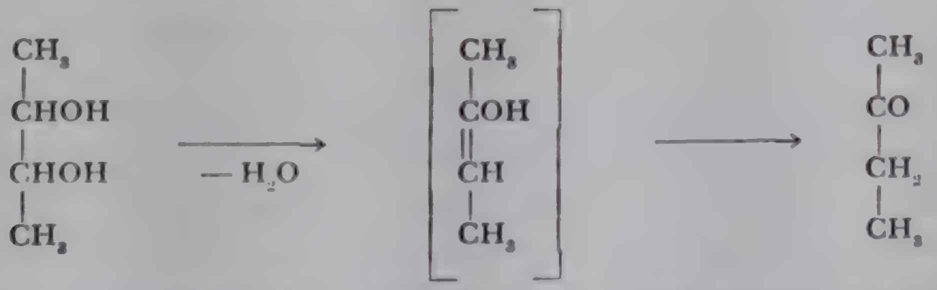
TABLE 3. COMPOUNDS READILY PREPARED FROM 2,3-BUTANEDIOL

Compound	Method of preparation	References
$CH_3{\cdot}CHOH{\cdot}CO{\cdot}CH_3$ Acetoin	Formed during fermentation when strongly aerated	111, 135, 191, 261
	Vapor-phase oxidation or dehydrogenation of 2,3-butanediol over copper catalysts	23, 53, 145, 157
$CH_3{\cdot}CO{\cdot}CO{\cdot}CH_3$ Diacetyl	Vapor-phase oxidation or dehydrogenation of 2,3-butanediol over copper catalysts	53, 116, 145, 157
$CH_3{\cdot}CO{\cdot}C_2H_5$ Methyl ethyl ketone	2,3-Butanediol heated with sulfuric acid, the diol being added continuously as volatile products distill	6, 169
	2,3-Butanediol vapor passed over dehydration catalysts, such as alumina, bentonite, or phosphorus pentoxide in a heated tube	10, 27, 50, 84, 153, 185
 $CH_3{\cdot}CH(O{\cdot}CO{\cdot}CH_3){\cdot}CH(O{\cdot}CO{\cdot}CH_3){\cdot}CH_3$ 2,3-Butanediol diacetate	2,3-Butanediol and sulfuric acid passed countercurrent to acetic acid in a heated column	82, 223
	2,3-Butanediol heated with acetic and sulfuric acids in distillation column, using an entrainer to remove water	151, 226

Compound	Method of preparation	References
 $CH_3\cdot\underset{O\cdot NO_2}{CH}\cdot\overset{O\cdot NO_2}{CH}\cdot CH_3$ 2,3-Butanediol dinitrate	D-(—)-2,3-Butanediol esterified by nitric acid, using sulfuric acid as catalyst	146
Polyester of 2,3-butanediol and phthalic acid	2,3-Butanediol heated with *o*-phthalic anhydride	28, 268
$CH_3\cdot CHOH\cdot CH{=}CH_2$ Methyl vinyl carbinol	2,3-Butanediol vapor passed over thorium dioxide under reduced pressure at 350°C	280
	Vapor of 2,3-butanediol, mixed with methyl ethyl ketone, water, and triethylamine, passed over a mixed catalyst at 235°C	85
$CH_3\cdot CO\cdot CH{=}CH_2$ Methyl vinyl ketone	Vapor of acetoin, mixed with water and triethylamine, passed over alumina-silica-tungstic oxide catalyst	85
$CH_2{=}CH\cdot CH{=}CH_2$ 1,3-Butadiene	2,3-Butanediol vapor passed over thorium dioxide at 350°C under reduced pressure	280
	Vapor of a mixture of 2,3-butanediol, water, methyl ethyl ketone, and triethylamine passed over mixed catalyst at 235°C	85
	2,3-Butanediol diacetate vapor passed through tube at 585° to 595°C	93, 151, 153, 222
	2,3-Butanediol diacetate vapor passed through heated tube packed with copper phosphate catalyst	196
 Methyl ethyl ketal of 2,3-butanediol	Continuous distillation of 2,3-butanediol from sulfuric acid under reduced pressure	169
 Formal of 2,3-butanediol	Distills when acidified fermentation solution is heated with formaldehyde	225

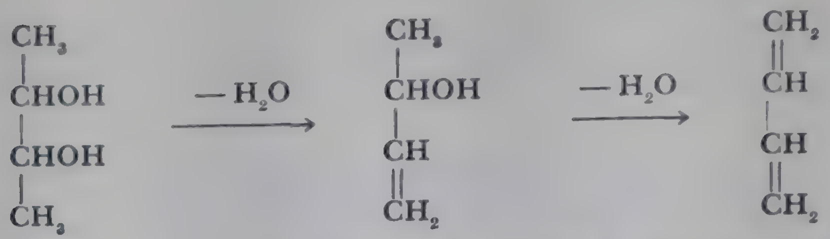
Compound	Method of preparation	References
 Butyral of 2,3-butanediol	Acidified fermentation solution extracted with butyraldehyde; the butyral is nearly all in solvent phase	248
 Cyclic carbonate of 2,3-butanediol	Reaction of 2,3-butanediol and phosgene	117
 Cyclic sulfite of 2,3-butanediol	Reaction of 2,3-butanediol and thionyl chloride	50, 208

The catalytic dehydration of 2,3-butanediol can lead to a variety of compounds. This type of reaction has received considerable attention because the chief initial impetus for the development of 2,3-butanediol fermentations was the production of 1,3-butadiene, the ultimate dehydration product of this diol. It is rather difficult to convert 2,3-butanediol to compounds with ethylenic double bonds by catalytic dehydration due to the strong tendency toward formation of methyl ethyl ketone instead, presumably by the reaction:



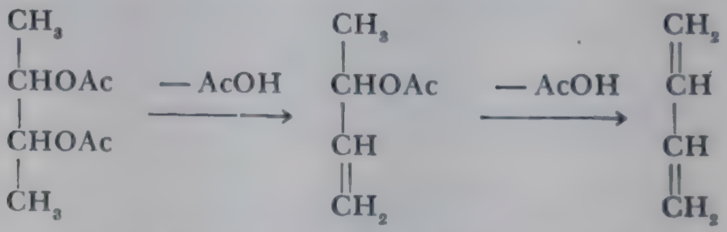
$$CH_3{-}CHOH{-}CHOH{-}CH_3 \xrightarrow[-H_2O]{} \left[CH_3{-}COH{=}CH{-}CH_3\right] \longrightarrow CH_3{-}CO{-}CH_2{-}CH_3$$

The majority of catalysts tested, acting in liquid or vapor phase, produce the ketone as the main product of the dehydration and this reaction could be used for the manufacture of methyl ethyl ketone if 2,3-butanediol were cheap enough to warrant it. However, conditions have been found which yield the more desirable ethylenic compounds, particularly methylvinylcarbinol and 1,3-butadiene.



This can be accomplished by using a thorium oxide catalyst at a pressure of about 65 mm and a temperature of 350°C. Single-pass conversions of 60% to butadiene or 80% to methylvinylcarbinol plus butadiene have been obtained with cumulative yields of the carbinol up to 90% of the theoretical value.[280] Some methyl ethyl ketone is obtained as a side reaction. Good yields of ethylenic compounds have also been claimed, using an oxide catalyst containing aluminum, tungsten, and silicon oxides.[85] The 2,3-butanediol is mixed with 10% methyl ethyl ketone, 50% triethylamine, and 20% water, the mixture vaporized and passed over the catalyst at 225° to 235°C. Butadiene and methylvinylcarbinol are the main products with little or no methyl ethyl ketone being formed. If acetoin is treated similarly (without addition of methyl ethyl ketone) a good yield of methyl vinyl ketone is obtained with 72% conversion in a single pass.

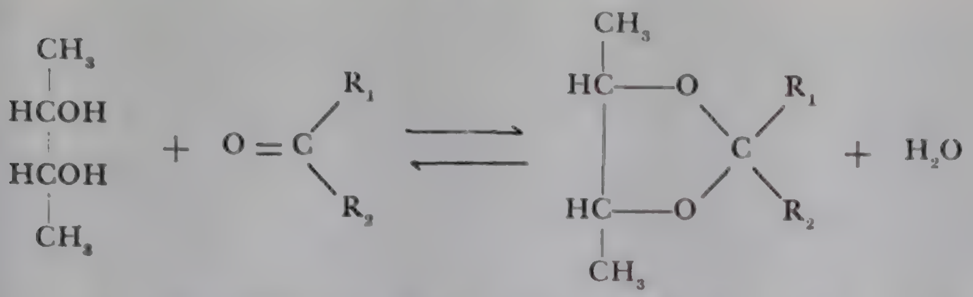
The most successful process for the manufacture of butadiene from 2,3-butanediol is pyrolysis of the diacetate:[93,151,153,222]



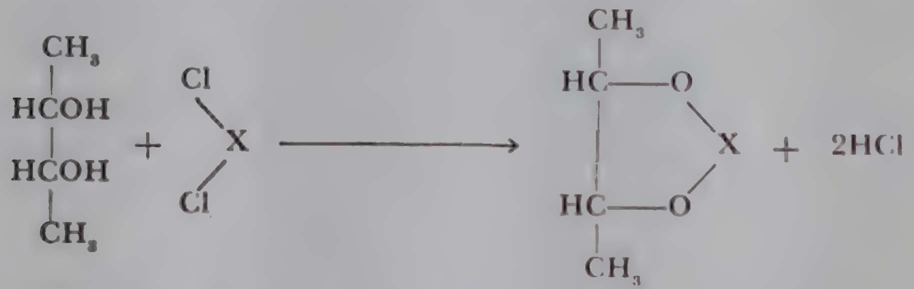
This process is known to work equally well with the *meso* and *levo* isomers and gives butadiene of over 99% purity. The reaction is usually accomplished by passing the vapor of the diacetate through an unpacked tube at 585° to 595°C. The acetic acid is recovered and used again. Yields of 82% are obtained on one pass with a cumulative yield of about 87% of theory.

2,3-Butanediol reacts readily with a number of compounds to give products which contain a five-membered ring with oxygen atoms in 1,3 positions. The three general types of reaction giving this ring are:

(a) Cyclic acetal formation[10,160,172] catalyzed by strong acids:

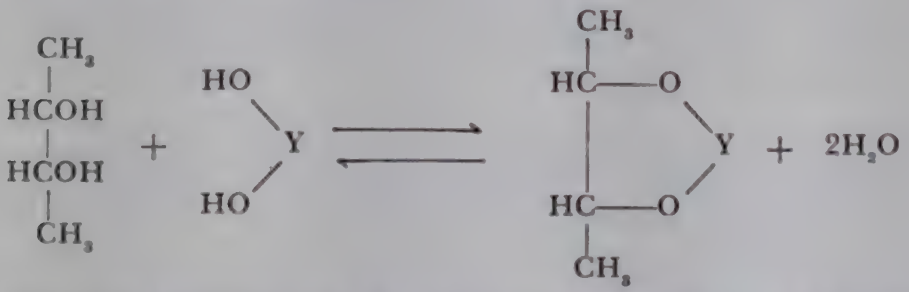


(b) Condensation with acid dihalides such as phosgene,[117] thionyl chloride,[50,208] or phenyl phosphorus oxydichloride:[255]



Good yields are obtained in these reactions, which occur spontaneously at room temperature.

(c) Condensation with dihydroxy acids such as arsenoacetic acid[66] and boric acid:[154,206]



At least two borates can be obtained from *meso*-2,3-butanediol.

There are no definite uses for these compounds. The cyclic acetals are of interest because of their ease of formation, which can be utilized in recovery of 2,3-butanediol from dilute aqueous solutions. They are volatile liquids not miscible with water which form diphasic, low-boiling azeotropes with the butanediol. If a fermentation solution is treated with formaldehyde and boiled, the formal of 2,3-butanediol distills as the azeotrope which separates into two layers.[225] The 2,3-butanediol is recovered by methanolysis of this compound. Alternatively, if the acidified fermentation solution (2 to 4% of 2,3-butanediol) is mixed with one-half volume of butyraldehyde, more than 98% of the 2,3-butanediol is extracted into the butyraldehyde layer as the butyral which is easily purified by distillation.[248] Hydrolysis of the butyral with dilute acid gives the 2,3-butanediol in high yields. These recovery methods would result in a cheaper production of the formal and butyral of 2,3-butanediol than of 2,3-butanediol itself and would be most attractive if direct uses could be found for these cyclic acetals. Catalytic cracking of these cyclic compounds gives chiefly methyl ethyl ketone from the part of the molecule contributed by 2,3-butanediol,[50,170] so that they do not appear promising as precursors of butadiene.

Both isomers of 2,3-butanediol form cyclic acetals readily but the (—) isomer reacts much better than the *meso* isomer with methyl ketones. If (—)-2,3-butanediol is dehydrated by heating with sulfuric acid, 95% of the theoretical amount of the methyl ethyl ketal can be obtained by continuous distillation.[169] Presumably, the methyl ethyl ketone forms and then reacts so rapidly with the excess diol that very little escapes in the free state, if the conditions are right.

These 1,3-dioxacyclopentane derivatives are good solvents, but like other ethers, they show a strong tendency to auto-oxidation. Antioxidants, such as hydroquinone, will control this tendency. Some of the lower members might serve as fuels for internal-combustion engines and some of the more viscous ones might be useful as plasticizers.

In addition to being a possible source for butadiene, 2,3-butanediol might be a source of monomers, such as methylvinylcarbinol and methyl vinyl ketone, for the plastics industry. It might also be used in place of glycerol in making resins of the polyester type. Some interesting studies have been reported on the polyphthalate[28,268] and this use might prove to be an important outlet for 2,3-butanediol. Perhaps these esters could be made from the butyral.

Physiological Properties

Neish[158] has reviewed the possible uses of 2,3-butanediol with reference to the pharmaceutical and food industries. Although, it is less toxic than ethanediol, it appears to be more toxic than 1,2-propanediol and is thus probably less desirable for pharmaceutical uses.

Acetoin and 2,3-butanediol are tolerated by rabbits in doses of 1 to 2 g per kg when administered subcutaneously.[173] Rats will tolerate about 3 g per kg, will recover from the effects of 6 g per kg, but are usually killed by 13 g per kg.[272] Acetoin disappears from the blood at a rate proportional to its concentration and at about the same rate as ethanol.[78] Only a small percentage is excreted unchanged in the urine,[78,173,272] but 5 to 25% may be excreted as 2,3-butanediol. Twelve to 14% of administered 2,3-butanediol is excreted in the urine unchanged, or in combination with glucuronic acid.

Administration of acetoin and 2,3-butanediol to rabbits significantly increased the biological acetylation of *p*-aminobenzoic acid; sodium acetate was without effect under the same condition.[59] This suggests that acetoin plays a role in metabolic acetylations. This is the only physiological use so far suggested to account for the presence of acetoin and acetoin-forming enzymes in animal tissues.

Diacetyl has antiseptic properties and a 0.1% solution will kill most pathogenic bacteria.[142] It will also inhibit the growth of the tubercle baccillus[14,16] and many nonpathogenic organisms[124] more effectively than benzoic acid.

ANALYTICAL METHODS FOR THE DETERMINATION OF 2,3-BUTANEDIOL, ACETOIN, AND DIACETYL

Lack of simple quantitative analytical methods has undoubtedly deterred bacteriologists from investigating the production of 2,3-butanediol, acetoin, and diacetyl by microorganisms. However, most of these difficulties have now been overcome and the investigator has a wide choice of methods available for use in analyzing fermentation solutions. Other compounds, such as ethanol, glycerol, acetic, formic, lactic, and succinic acids, are usually present and must be taken into consideration. Detailed methods for these need not be listed here, but have been published.[163] The authors recommend the use of microdiffusion[281] for ethanol, partition chromatography[161] for the acids, and colorimetric estimation of the formaldehyde formed by periodate oxidation[125] for glycerol. The specificity of the glycerol determinations can be greatly increased if it is first separated from interfering substances by chromatography.[162]

Methods for the Determination of Diacetyl and Acetoin

Diacetyl has never been reported in very large amounts in fermentation solutions, but its determination merits consideration since many methods proposed for the estimation of acetoin and 2,3-butanediol depend on their conversion to diacetyl. Many of the methods for determining diacetyl depend on converting it to dimethylglyoxime by treating with an excess of hydroxylamine. There are numerous ways of estimating dimethylglyoxime.[11,55,94,96,183,199,220,240,246,274] There are also three direct colorimetric methods for determining diacetyl,[48,64,119,195,270] and there is a polarographic method.[78]

Several methods have been proposed for the determination of acetoin. It can be readily oxidized to diacetyl in high yields by heating with ferric chloride and the yields are almost quantitative if a mixture of ferric and ferrous salts is used.[113,134,183,270] This reaction can be conducted in a tightly stoppered tube.[270] The diacetyl is then distilled from the oxidation mixture and may be estimated by any of the available methods.

Other methods for the determination of acetoin depend on its separation from interfering substances, such as sugars, by distillation of the dilute aqueous solution. Acetoin behaves somewhat like the volatile fatty acids, a definite fraction of it distilling along with a definite fraction of the water.[128] Most of the important methods utilize this property in order to attain some degree of specificity. The acetoin in the distillate may be determined iodimetrically by oxidation with alkaline iodine,[128] acidimetrically by titration of acetic acid formed on oxidation by periodate,[33,159] by measuring the reducing power with alkaline copper sugar reagents,[33,133,263] by titration of the hydrochloric acid released on reaction of acetoin with hydroxylamine hydrochloride,[190] and gravimetrically as *p*-nitrophenylosazone.[180] Acetoin and diacetyl can be determined by analyzing the first half of the distillate colorimetrically, using the creatine-α-naphthol reaction.[12,163,187,270] If the color is allowed to develop for 1 hour under prescribed conditions[270] then equal weights of diacetyl and acetoin give the same intensity of color.[163] The diacetyl can be estimated separately, using the hydroxylamine-urea color reaction[272] and the acetoin obtained by difference. This is the method used by the authors.[163] Acetoin is difficult to recover quantitatively in an ether extract.

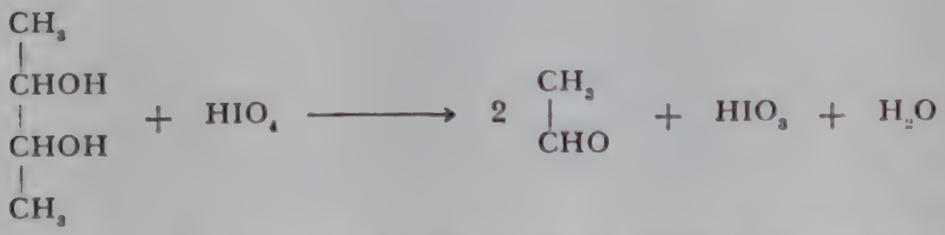
However, it may be quantitatively separated from many other substances by partition chromatography and determined by the creatine-α-naphthol color reaction.[163]

Methods for the Determination of 2,3-Butanediol

2,3-Butanediol is usually the major product of the fermentations discussed in this chapter. The numerous procedures for its determination fall into two general classes.

(a) Methods based on oxidation to diacetyl by bromine and ferric chloride belong to the first group.[83,94,95,96,109,113,134,192,221,252] The diacetyl formed is estimated by previously outlined procedures. Methods based on the measurement of diacetyl are specific, but not very accurate since the yields are not quantitative. Furthermore, these methods are difficult to develop into rapid, precise procedures such as are useful in routine analysis.

(b) The second group includes those methods which are based on oxidation to acetaldehyde by periodic acid:



$$CH_3CHOHCHOHCH_3 + HIO_4 \longrightarrow 2\,CH_3CHO + HIO_3 + H_2O$$

In these methods, either the acetaldehyde formed or the periodate consumed may be measured. Obviously, it is more specific to measure the acetaldehyde, but the titration of periodate is very accurate and may be preferable in the absence of interfering substances.[68,200] If the diol is first extracted from a neutral aqueous solution by ether[18,100,163] or butanol,[194] the interference due to sugars is eliminated and the specificity increased. The diol in *A. aerogenes* and *B. polymyxa* fermentations can be determined in this fashion, but the glycerol present in *B. subtilis* fermentations causes a + 10% error unless correction is made.[163]

The most useful methods for the estimation of 2,3-butanediol are probably those based on the measurement of the acetaldehyde formed by periodate oxidation. They are fairly specific though not as specific as those based on the measurement of diacetyl. However, it is possible to determine 2,3-butanediol in the presence of equal or greater amounts of sugar or glycerol by some of these procedures.

In order to do this, it is necessary to separate the acetaldehyde formed by periodate oxidation from the formaldehyde formed at the same time or to use a colorimetric method in which formaldehyde does not interfere. The blue color developed on treating dilute acetaldehyde solutions with piperazine and sodium nitroprusside permits rapid estimation of 2,3-butanediol, since it can be applied in the presence of formaldehyde and excess periodate.[54] Determinations accurate to within 2 to 3% can be made, in spite of the instability of the color, if a photoelectric colorimeter is used. This method can be applied to diluted fermentation solutions, ether extracts, or butanol extracts[163] with no appreciable interference from equal amounts of glycerol or glucose. If the glycerol concentration is much higher than the diol concentration the reaction can be carried out in a microdiffusion unit and in this way, 2,3-butanediol can be estimated in the presence of fifty times as much glycerol.[163]

The volumetric determination of acetaldehyde absorbed in hydroxylamine hydrochloride[71] or bisulfite[100,186,205,272,282] solutions may yield accurate results, but precautions must be taken to exclude formaldehyde. This makes a distillation necessary[100,186,203,205,227,239,272] in the procedure, if much glycerol is present. However, microdiffusion[163,280] into bisulfite can be used for the volumetric determination of 2,3-butanediol in the presence of an equal amount of glycerol.

The specificity of methods based on periodate oxidation is increased by introducing some procedure for the separation of 2,3-butanediol from other substances that react with periodate. This is often done by steam distillation of the solution after addition of sodium carbonate or by continuous extraction with ether. The extraction with ether may be accomplished quantitatively in 2 hours, if small extractors of 5 ml capacity are used[163] and the organic acids can then be extracted from the same sample after acidification with hydrochloric acid. For this reason, ether extraction is preferred over distillation or butanol extraction if a complete analysis is to be made.

These techniques do not give a sharp separation of the 2,3-butanediol from glycerol, and acetoin is still present, though not necessarily in quantitative amounts. A method based on fractional steam distillation[186] has been proposed which gives an almost complete separation of acetoin and 2,3-butanediol and appears to have a high degree of specificity. Perhaps the most specific method is based on the use of partition chromatography on celite-water columns.[162,163] This gives a sharp, quantitative separation of acetoin, 2,3-butanediol and glycerol, and permits the quantitative determination of each in separate fractions.

The selection of the method to be used for the determination of 2,3-butanediol or acetoin depends on the sample being analyzed. If very little glycerol is present, as in the *A. aerogenes* and *B. polymyxa* fermentations, the diol may be extracted with ether or butanol and determined by titration of the periodate consumed. The most rapid methods for analysis of large numbers of cultures[163] are the colorimetric procedures based on the use of creatine-α-naphthol for acetoin,[270] and piperazine-sodium nitroprusside for 2,3-butanediol.[54] If acetoin and 2,3-butanediol are the major products, these reactions can be run directly on the diluted fermentation solutions at the rate of about one hundred determinations a day per worker. However, if they are minor components of the mixture being analyzed, or if interfering substances are present, they must be separated from the mixture by distillation or ether extraction respectively before developing the color, since substances may be present which modify the intensity of the color. The 2,3-butanediol value must be corrected for the acetoin since 1 mol of acetoin gives 1 mol of acetaldehyde on periodate oxidation.

NATURAL OCCURRENCE OF 2,3-BUTANEDIOL, ACETOIN, AND DIACETYL

Acetoin and 2,3-butanediol are found in small amounts in many common materials, particularly foodstuffs (see Table 4). Usually, they are formed as a result of the action of microorganisms, but it is interesting to note that both are also present in the tissues of higher plants and animals. Foods and beverages which have been subjected to the action of yeasts during their manufacture invariably contain 2,3-butanediol and often acetoin as well. The amount found in wines is quite variable and different types of wines can be distinguished by measurement of their 2,3-butanediol contents.[114] Dry wines, to which no spirits have been added, contain about one hundred to two hundred times as much ethanol as 2,3-butanediol, while fortified sweet wines contain five hundred to twenty-five hundred times as much. Normally, there is not much acetoin in wines, although some yeasts in pure culture form quite appreciable amounts.[illegible] The apiculate yeasts form aceto cannot reduce it to 2,3-butanediol, while the elliptical yeasts are always present, reduce acetoin readily.[illegible] Therefore, is usually found in very small amounts. When vinegar i from wine, the acetoin content increases without much cha the 2,3-butanediol content, indicating a possible synthesis of from ethanol by the acetic acid bacteria.[186]

TABLE 4. NATURAL OCCURRENCE OF 2,3-BUTAN
ACETOIN AND DIACETYL

Source	Quantity	Refere
Beer	18 to 26 ppm acetoin; 10 to 51 ppm 2,3-butanediol	203
Wines	Up to 1,550 ppm 2,3-butanediol and up to 20 ppm acetoin. Ciders may have 300 to 400 ppm acetoin	53, 7 82, 11 200, 20
Vinegar	Up to 300 ppm 2,3-butanediol; up to 800 ppm acetoin	73, 1
Bread	Up to 5 ppm acetoin; less than 10 ppm 2,3-butanediol	261, 2
Butter	Up to 4 ppm acetoin and about 1.7 ppm diacetyl	184, 1
Cheese	Diacetyl up to 3.4 ppm, but usually less than 0.5	56, 5
Coffee	10 to 15 ppm of both acetoin and diacetyl in freshly roasted coffee. Diacetyl changes to acetoin as coffee stales	98
Blood and urine of higher animals	About 1 ppm acetoin and 4 ppm 2,3-butanediol in blood—less in urine	146, 2
Seedlings of higher plants	Up to 91 ppm acetoin and 28 ppm 2,3-butanediol	189

Diacetyl has a strong odor and is believed to be the ch ponent of butter aroma[illegible] and to contribute to the of other foods. It contributes to the aroma of tobacco, s roasted coffee,[98] honey, and dark beer.[illegible] Acetoin is also these products, usually in higher concentration than diacety it is easily oxidized to diacetyl, it may be regarded as a less reserve supply of aroma. A great many papers have been p on the relation of diacetyl to the aroma of butter. It is found in a concentration of 1 to 2 ppm,[illegible] although a conce of 5 ppm is considered more palatable. It may be used for tter or margarine and has also preservative value. For example, to 12 ppm of diacetyl will preserve maragarine against develop- nt of "off flavors" better than 0.2% benzoic acid.[221] In addition, it es a butterlike aroma when added in the recommended quantity 6 ppm.

Bacterial cultures are used in the manufacture of butters of high oma. Milk is acidified with citric acid and bacteria, such as *eptococcus citrovorus* and *S. lactis*, are allowed to develop. These ment the sugar and citric acid, producing 2,3-butanediol, toin, and diacetyl.[194] When a properly ripened culture is added the pasteurized cream, it enables the manufacturer to produce a tter with better aroma and keeping qualities than untreated tter.[87] Aeration favors production of diacetyl and acetoin rather an the nonaromatic 2,3-butanediol,[22,88] which is usually present o in butter cultures.

A great many species of bacteria produce acetoin and/or 2,3- tanediol. The tests for acetoin are widely used by bacteriologists d are of particular value in classifying the Enterobacteriaceae d Bacillaceae.[17,137] In an investigation on one hundred seventy- ır strains of anaerobic bacteria, belonging to sixty-four species, was found that 72.4% formed acetoin[138] and this was considered be a valuable feature to use in their classification.

Many of these organisms probably give quite small amounts of etoin and 2,3-butanediol. This is true of all the species of *Clostrid- n* which have been analyzed carefully. *Cl. felsineum* and *Cl. tyricum* produce acetoin, but are unable to reduce it to -butanediol.[35] In this respect, they are similar to the apiculate sts mentioned before.

Other organisms, such as the *Aerobacter* and *Serratia* species, *polymyxa*, *B. subtilis* and *Pseudomonas hydrophila* will ferment re than half of the sugar to 2,3-butanediol and acetoin. It is eresting that these 2,3-butanediol bacteria should be found in e families, showing such marked morphological differences.

Many of the organisms known to produce acetoin have not been ed very thoroughly and under optimum conditions might form stantial amounts of acetoin and 2,3-butanediol. Most cultures cetoin-producing bacteria normally contain 2,3-butanediol, often higher concentrations than the acetoin. There are probably ny strains of bacteria which are of potential value for 2,3- anediol production. Any organism which shows a positive test for acetoin is worth testing further, provided it will ferment at a reasonably rapid rate. In the following pages, only a few species, which have been investigated rather extensively, will be considered.

BACTERIOLOGICAL METHODS

The most intensive studies for 2,3-butanediol production have been on species of *Aerobacter* and *Serratia* of the Enterobacteriaceae and *Bacillus polymyxa* and *B. subtilis* of the Bacillaceae. No attempt will be made to deal with problems of nomenclature, nor to list the many synonyms of the species mentioned. The system of nomenclature recommended by Bergey's *Manual of Determinative Bacteriology,* Sixth Edition,[17] is followed here.

Aerobacter aerogenes (Kruse) Beijerinck

Organisms of this genus are universally distributed in nature, being very abundant in soil and plant products and also occurring in the intestines of man and animals. Fresh vegetables, such as celery, spinach, carrots, lettuce, yams, onions, and potatoes, have been found most useful for seeding enrichment cultures. Cultures are also readily available from many culture collections.

Aerobacter cultures may be maintained by transfer on slants of the medium suggested by the Northern Regional Research Laboratory which contains, per liter, 5.0 g tryptone, 1.0 g glucose, 5.0 g yeast extract, and 15.0 g agar. Starter cultures for *Aerobacter* fermentations were developed[264] by growing the organism for 8 to 20 hours at 30°C in an unagitated, unaerated medium which contained, per liter, 50 g glucose, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.60 g KH_2PO_4, 5.0 g $CaCO_3$, and 0.20 g urea. All ingredients were mixed together, except the urea, and the medium was sterilized at 121°C for 30 minutes; then 1 ml of sterile 20% urea solution was added for each 100 ml of basic medium.

Serratia marcescens Bizio

Species of this genus are small, aerobic rods, usually producing a bright-red or pink pigment on agar and gelatin. Cultures have been isolated from water, soil, milk, foods, silk worms and other insects, but no dependable enrichment techniques are available for obtaining them. Most fermentation studies have been made on cultures available from collections.

Bacillus polymyxa (Prazmowski) Migula

The classification, morphological and biochemical properties of *B. polymyxa* have been described[61,197,229] and also procedures for isolation of strains having active fermentation properties.[131] Rich garden soil is a very dependable source of inoculum for enrichment cultures, though other natural sources, such as soil water, and fresh or decaying vegetables, may also be used. It was found that pasteurization of the original inoculum in water at 80°C for 10 minutes is very important. The pasteurized suspension may be inoculated in 1 ml amounts into tubes of lactose or 1.5% starch media and incubated at 30°C. Streak plates are made in 2 or 3 days from cultures showing gas production. Neutral red agar, plain starch-peptone, or starch yeast-extract agar are all satisfactory media for this purpose. Presence of neutral red, which stains *B. polymyxa* colonies pink or red, aids in their selection from other species. Slime formation and the presence of gas bubbles, as well as a pleasant fruity odor, are additional characteristics which aid in the selection of *B. polymyxa*. However, great differences in colony form, consistency, surface, and elevation occur between isolates and the final selection depends on biochemical characteristics.

Stock cultures in active use may be carried on agar slants, containing 5% whole wheat, or 2% soluble starch, containing 0.5% yeast extract and 0.5% calcium carbonate.

Bacillus subtilis (Ford strain)

There is still much uncertainty as to the taxonomic position of the Ford strain of *B. subtilis*. Gibson[76] regards it as a distinct species, *B. licheniformis*, but Smith, Gordon, and Clark[229] and Bergey's Manual[17] do not distinguish between it and the recognized type culture, the so-called Marburg strain. It has been found[22,165] that under anaerobic conditions, the Ford strain produces glycerol and 2,3-butanediol whereas the Marburg strain is a strict aerobe and does not produce glycerol.

B. subtilis is very widely distributed in the soil and decomposing organic matter. Enrichment cultures of dried hay, straw, or soils rich in organic matter may be pasteurized at 90 to 100°C for 10 to 30 minutes and samples used to inoculate tubes of nutrient media. After incubation at 35°C for 24 hours, cultures of *B. subtilis* and related species may be picked from streak plates. These cultures are then differentiated by biochemical tests as outlined by

Smith et al.[229] After this, it is necessary to carry out strictly anaerobic fermentations on all *B. subtilis* types to differentiate Ford and Marburg strains.

FERMENTATION OF CARBOHYDRATES BY 2,3-BUTANEDIOL BACTERIA

Types of Fermentation

The 2,3-butanediol bacteria may be defined as those in which 2,3-butanediol is the major fermentation product (i.e., usually 20% or more based on the weight of the sugar fermented). The first species to be placed in this group was *Aerobacter aerogenes* which was found, in 1906, to give good yields of 2,3-butanediol.[89] This organism grows well anaerobically in a calcium carbonate-buffered medium, containing sugars, and forms 2,3-butanediol according to the equation:

$$C_6H_{12}O_6 \longrightarrow CH_3{\bullet}CHOHCHOH{\bullet}CH_3 + 2\,CO_2 + H_2$$

Other 2,3-butanediol bacteria, such as *Bacillus polymyxa,*[61] *Aerobacter cloacae,*[247] *Aerobacter faeni,*[30] *Aerobacter indologenes,*[149] *Pseudomonas hydrophila,*[231] and *Serratia plymuthicum,*[167,192] also ferment carbohydrates according to this general equation under appropriate conditions. However, it is evident that 2,3-butanediol bacteria which do not form molecular hydrogen must have some other way of disposing of the two hydrogen atoms left over when 2,3-butanediol is made from glucose. Some bacteria of the genus *Serratia* accomplish this, under anaerobic conditions, by production of formic acid:[167,192]

$$C_6H_{12}O_6 \longrightarrow CH_3{\bullet}CHOH{\bullet}CHOH{\bullet}CH_3 + CO_2 + HCOOH$$

However, the group of bacteria represented by Ford's type of *Bacillus subtilis* form glycerol:[21,166]

$$3\,C_6H_{12}O_6 \rightarrow 2\,CH_3{\bullet}CHOH{\bullet}CHOH{\bullet}CH_3 + 2\,CH_2OH{\bullet}CHOH{\bullet}CH_2OH + 4\,CO_2$$

All these bacteria are heterofermentative and carry out the well known lactic acid or alcoholic fermentations as well as 2,3-butanediol fermentations:

$$\begin{aligned} C_6H_{12}O_6 &\longrightarrow 2\,C_2H_5OH + 2\,CO_2 \\ C_6H_{12}O_6 &\longrightarrow 2\,CH_3{\bullet}CHOH{\bullet}COOH \end{aligned}$$

The relative importance of these reactions in the different species of 2,3-butanediol bacteria is shown in Table 5. These figures were obtained by culturing the bacteria anaerobically in a calcium carbonate-buffered medium, containing 5% glucose with accessory nutrients, usually supplied by 0.5% yeast extract. The actual weight of products obtained under these conditions is shown in Table 6 for the four most interesting species. These organisms should not be compared solely under these conditions, since all 2,3-butanediol fermentations are sensitive to changes in conditions. For example, *Aerobacter aerogenes* gives the lowest yield of useful products anaerobically, but it is probably the best organism to use for production of 2,3-butanediol, since it grows and ferments more vigorously than the other organisms and the yield of 2,3-butanediol can be increased to 40 lb of 2,3-butanediol per 100 lb of glucose fermented by moderate aeration.[70,186] Thus under readily attainable conditions, it will produce more 2,3-butanediol than the other organisms whose yield is not raised by aeration as much. *B. polymyxa* is of interest since it will ferment starchy matter directly and produces pure (—)-2,3-butanediol. *S. marcescens* produces considerable amounts of formic acid, but it is doubtful if this could be economically recovered on a commercial scale. *B. subtilis* is interesting in that it gives good yields of glycerol as well as of 2,3-butanediol and the sum of these two products is considerably greater than the theoretical amount of diol produced by the other species.

TABLE 5. RELATIVE IMPORTANCE OF THE VARIOUS FERMENTATION REACTIONS IN SPECIES OF BACTERIA PRODUCING 2,3-BUTANEDIOL

Organism	Percentage of sugar dissimilated by fermentations producing				
	Diol-hydrogen	Diol-formic acid	Diol-glycerol	Ethanol	Lactic acid
Aerobacter aerogenes	52	2	—	23	2
Pseudomonas hydrophila	56	—	—	26	12
Bacillus polymyxa	68	—	—	33	—
Serratia plymuthicum	49	2	—	23	14
Serratia marcescens	1	55	1	27	6
Serratia indica	1	50	1	22	5
Serratia anolium	1	48	1	21	4
Bacillus subtilis (Ford's Type)	—	—	84	3	12

TABLE 6. MAJOR PRODUCTS OF ANAEROBIC 2,3-BUTANEDIOL FERMENTATIONS

Product	Pounds per 100 lb glucose fermented[a]			
	Bacillus polymyxa	*B. subtilis* (Ford's Type)	*Aerobacter aerogenes*	*Serratia marcescens*
2,3-Butanediol	32.6	27.5	24.0	32.0
Ethanol	17.1	2.0	17.2	11.8
Glycerol	—	29.4	—	—
Lactic acid	—	11.6	2.4	5.0
Formic acid	—	0.3	0.8	12.3
Carbon dioxide	48.2	26.9	43.8	29.6
Hydrogen	0.78	—	0.47	—
	98.68	97.7	88.67	90.7

[a] Acetic acid, succinic acid, and assimilated carbon account for the remainder of the glucose used.

Effects of Aeration

Aeration of the cultures during the fermentations has a marked effect. In the presence of oxygen, all these organisms tend to ferment according to the equation:

$$C_6H_{12}O_6 + \frac{1}{2}O_2 \longrightarrow CH_3{\cdot}CHOH{\cdot}CHOH{\cdot}CH_3 + 2\,CO_2 + H_2O$$

Thus under aerobic conditions *S. marcescens* gives very little formic acid, *B. subtilis* yields hardly any glycerol, and hydrogen production by the other species is lowered to varying extents, depending on the organism.

In considering the effect of aerating these fermentations, it must be realized that the over-all effect on a complex system is being observed. A suitable medium is inoculated with a comparatively small number of cells. These cells multiply, reach a peak population, and eventually die off. During this period, the sugar is fermented. Aeration may affect both the rate of growth and the rate of fermentation per cell, as well as the relative concentration or activities of the enzymes produced by the cells. If air or oxygen is passed through a fermenting medium, it can act by agitating the medium, thus aiding neutralization of acids by the calcium carbonate, or it may affect the fermentation by removing the carbon dioxide or by acting as a hydrogen acceptor. Some experiments have been designed to separate these variables and it is possible to make certain inferences concerning the results observed when a stream of air is passed through a 2,3-butanediol fermentation.

In general, aeration increases the yield of the C_4 compounds (2,3-butanediol and acetoin) as compared with the other products.

[70,186,211,263] Experiments with *B. polymyxa* have shown that there s a gradual decline in the proportion of the carbohydrate-forming $_4$ compounds during anaerobic fermentation and that aeration aises the yield and prevents this decline.[211] Oxygen retards the ethanol formation by *B. polymyxa* and it is possible to change the liol to ethanol ratio from 1.4 to 3.6 if it is used.[2] *A. aerogenes* acts similarly and gives a greatly increased yield of 2,3-butanediol when erated.

In addition to changing the yields of the products, aeration lso affects the over-all rate of fermentation. Oxygen is not necesary to obtain these increased rates if *A. aerogenes* and *B. polymyxa* re used; gases, such as nitrogen or hydrogen, passed through fermenting solutions also cause an increased rate.[2,217] Carbon dioxide s not suitable for this purpose and it is possible that the beneficial effects of the other gases is due to the removal of carbon dioxide. This view is supported by the fact that more rapid fermentations ccur with a high surface to volume ratio or when fermentations re run under reduced pressure.[4,102]

It is safe to say that aeration should be beneficial to most 2,3-utanediol fermentations, except *B. subtilis* fermentations, where t greatly reduces the yield of glycerol.[21,166] The exact amount of eration required for any fermentation has to be worked out on pilot-plant scale for the organism and medium to be used.

Effect of pH on the Fermentation

Due to the sensitivity of bacteria to variations in the environment, there is still much work to be done in determining optimum onditions for the various fermentations. A good example of the need for close control of conditions may be seen in the effects of the ydrogen-ion concentration of the medium on the rate and course f the fermentation. This has been shown in experiments on ermentations controlled to within 0.05 pH unit by automatic addition of ammonium hydroxide.[171] The effect of pH on the rate of arbohydrate breakdown by the four most interesting species of acteria are shown in Figure 2. They all show an optimum rate ear pH 7, but *B. subtilis* and *S. marcescens* are more sensitive to cid. These four species of bacteria produce 2,3-butanediol well at H 6.2 or below, but above this pH, the yield drops rapidly until at H 7.6 little or none is formed (see Figure 3). Earlier work on *B. olymyxa*[3] and *A. indologenes*,[148] using manual control, had indi-

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cated that lower diol yields were obtained at high pH valu
that a critical point occurred at pH 6.3. This seems to b
for the five species tested so far. The reason for this rapid d
in the yield is not known, but it seems to be at least partly
the effect of pH in altering the enzymic constitution of th
which are grown in the medium[56]. In addition, it is probab
pH affects the mature cells as well.

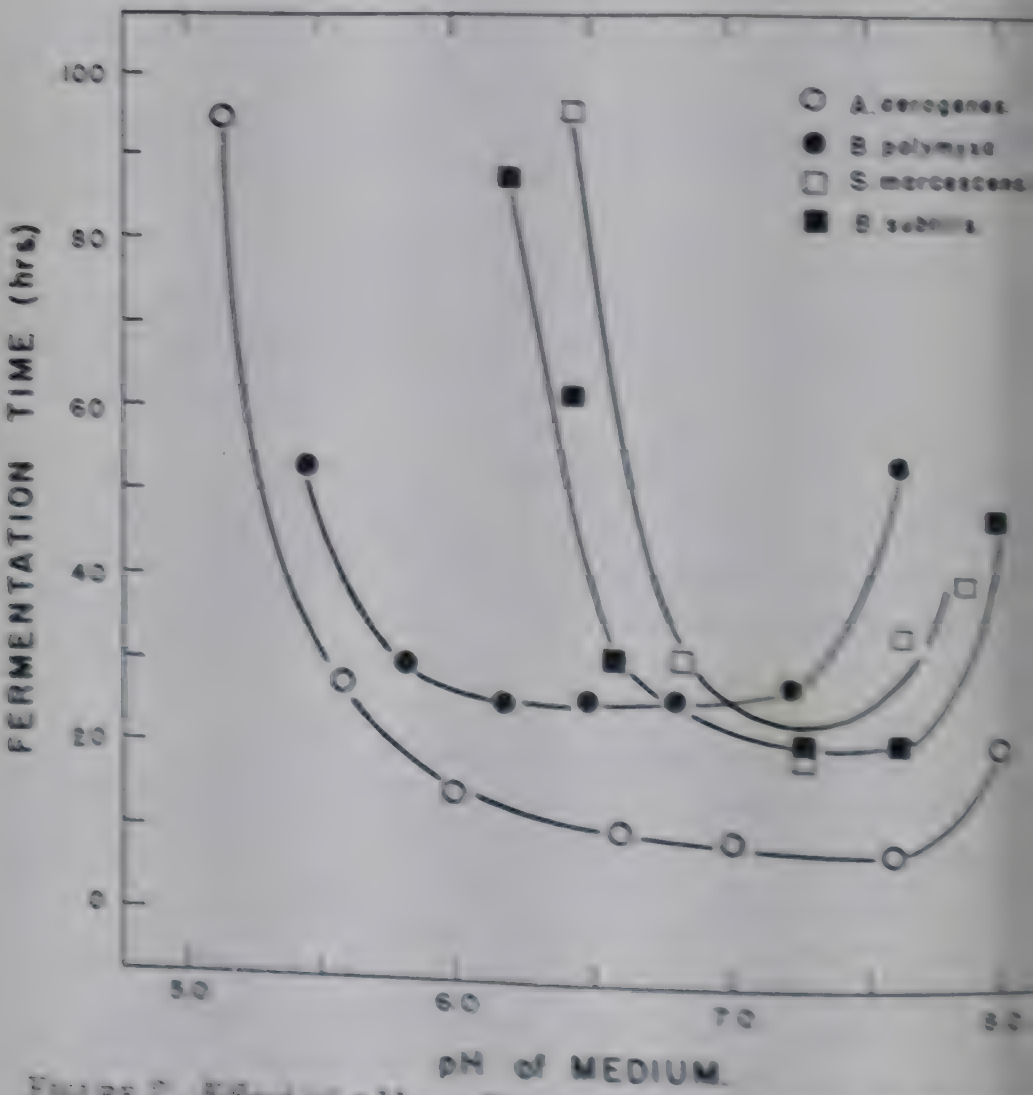
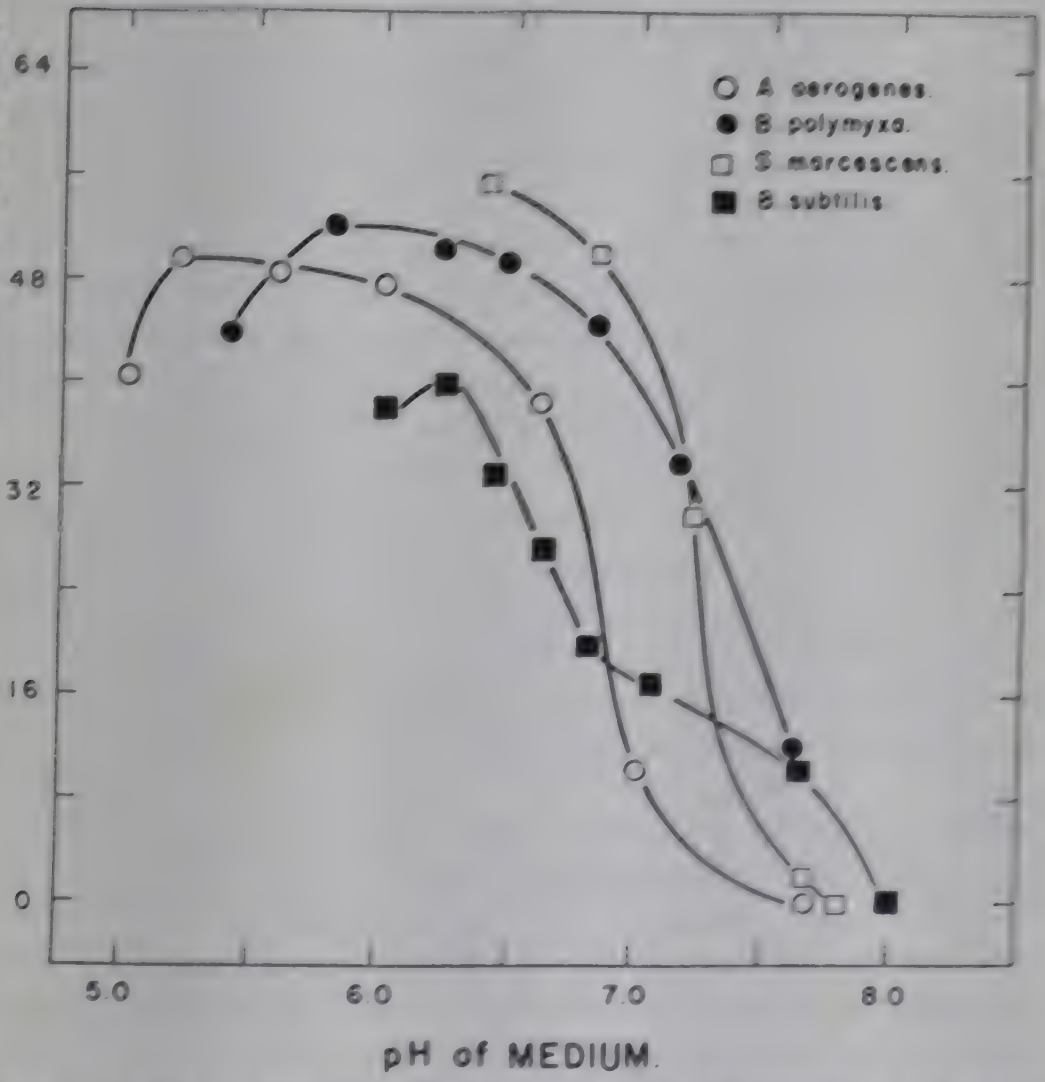


FIGURE 2. *Effect of pH on Rate of Dissimilation of 5% Gl*

The failure of these bacteria to form much carbon diox
hydrogen in alkaline media is remarkable. Formic acid is
instead and the diol is replaced largely by acetic acid. There
to be a definite relation between the yields of acetic acid a
butanediol. When acetate is added to a fermenting *Aerobac*
ture at a pH favorable for 2,3-butanediol fermentation, the

duced and the 2,3-butanediol yield is increased enough to ac-
nt for all the acetate disappearing (diol increased from 61 mols
8 mols per 100 mols of glucose fermented). This may result in
st complete suppression of the hydrogen formed.[168, 204] Similar
lts have been obtained with *B. polymyxa.*[207, 208] Propionate acts
larly to acetate in increasing the diol and decreasing the hydro-



re 3. *Effect of pH on Production of 2,3-Butanediol by Four Species of Bacteria*

ield while succinate increases the diol and formic acid yields,
decreasing hydrogen production. Since propionate is reduced
opanol, this suggests that these acids act by a mechanism similar
at of other hydrogen acceptors in raising the yield of 2,3-

butanediol. However, ferric hydroxide is reduced by *B. polymyxa*, without increasing the diol yield.[207] Since reduction of acetates tends to make the medium alkaline, they might be useful buffering agents for controlling the pH of 2,3-butanediol fermentations on an industrial scale. The neutralizing agent consumed would then be recovered as the product. This possibility seems to have been overlooked by most investigators who have studied these fermentations from an industrial point of view.

If the acids formed as by-products by most of these bacteria are not neutralized, the pH falls too low for vigorous growth and fermentation. Calcium carbonate has been the most widely used buffering agent; it can be added in excess and goes into solution as it is required. It usually buffers in the range of pH 5.6 to 5.9 which is favorable for 2,3-butanediol formation but low enough to limit the rate of the fermentations. The best pH for carrying out 2,3-butanediol fermentations is about 6.2, since good diol yields are still obtained and the reaction is considerably faster than in calcium carbonate fermentations.[171,269] It is necessary to use an automatic pH-control system to achieve these conditions. In this case, ammonia is a good neutralizing agent. *B. polymyxa* fermentations on a sucrose medium are five times as fast when ammonium hydroxide is used as when sodium or potassium hydroxide is used as a neutralizing agent. The optimum conditions for anaerobic fermentation of sucrose by *B. polymyxa* are believed to be in a medium containing 8% sucrose at 33°C, controlled at pH 6.2 ± 0.05 by automatic addition of ammonium hydroxide. The fermentation is complete in 30 hours giving 130 mols of diol and 122 mols of ethanol per 100 mols of sucrose fermented.[269] It is likely that the same conditions would be suitable for *Aerobacter aerogenes*, although a temperature of 35°C is probably better.[70] Aeration under these conditions will raise the yield of 2,3-butanediol but decrease the rate of the fermentation as far as *B. polymyxa* is concerned.

Although most large-scale work on 2,3-butanediol fermentations has been done with calcium carbonate as a buffering agent, it appears that ammonia has the advantages of lower cost, inherent sterility, greater range of control, ease of addition in the liquid or gaseous state, greater nutrient value, and acceleration of fermentation.[3,269]

Effect of Sugar Concentration

Increasing the initial sucrose concentration increases the yield of 2,3-butanediol in *B. polymyxa* fermentations.[73,193] Under aerobic conditions on a medium containing 10% sucrose with pH controlled at 6.2 a diol + acetoin to ethanol ratio of 11 to 1 may be obtained with *B. polymyxa*.[269] A 6% solution is almost completely fermented in 71 hours, giving 104 mols of 2,3-butanediol, 48.6 mols of acetoin and 40 mols of ethanol per 100 mols of sucrose.

Aerobacter aerogenes is capable of fermenting fairly high concentrations of sugars, if they are added gradually. Experiments have been performed in which a concentrated solution of glucose was fed into a fermentation at such a rate as to keep the sugar concentration in the fermentor close to 3%. In this way, sugar concentrations corresponding to 26.5% were fermented in 108 hours, giving 2,3-butanediol concentrations up to 9.8%.[62,186]

Nutrient Requirements

Organisms of the *Aerogenes* group *(Aerobacter, Serratia)* have fairly simple nutrient requirements and will ferment well on a synthetic medium containing only sugars and mineral salts. *B. subtilis* and *B. polymyxa* need a complex nitrogen source in order to grow and ferment well. However, *B. polymyxa* is able to ferment starchy substrates, such as whole wheat or corn mashes,[102,121,130,131,266] directly, whereas a preliminary hydrolysis is necessary before they can be fermented by *Aerobacter* strains.

It is more desirable from a chemurgic and engineering viewpoint to separate the starch from the other components of grain before fermentation. Consequently, investigations have been conducted on the fermentation of starch by *B. polymyxa*.[69,103,104,121] This organism requires biotin and complex nitrogen sources to ferment well.[105]

Good fermentations of cornstarch were obtained by Kooi, Fulmer, and Underkofler[121] with a medium containing 0.5% of corn gluten, 7.5% of cornstarch, and 0.0069% of potassium permanganate. Addition of malt sprouts or brewers' yeast as nutrients raised the yield of 2,3-butanediol. The resulting fermentation solution is more readily filtered than a whole-corn mash fermentation and valuable by-products are recovered in preparing the cornstarch.

Wheat starch is a poor medium for fermentation and su ments have to be added. In this case, the gluten is not stimula but the bran and shorts are. A 1% addition of malt sprouts the most effective supplement for wheat starch with shorts. Cerogras, alfalfa, soybeans, yeast extract, and corn-steep liqu decreasing order of effectiveness. It is possible to ferment pu wheat starch by some strains of this organism provided isole tyrosine, glycine, methionine, and asparagine are added. nutritional requirements of different strains vary somewhat some amino acids are inhibitory in concentrations above 0. Potassium stimulates 2,3-butanediol production from starch increases the diol to ethanol ratio. Phosphate also stimulates production, especially in the presence of potassium.[illegible] The duction of 2,3-butanediol by *A. aerogenes* is also stimulate phosphate as well as by peptone.[illegible]

In addition to fermenting glucose, fructose, and sucrose 2,3-butanediol bacteria will also ferment xylose and m tol.[illegible] On each of these substrates, 2,3-butanedi formed, although usually in lower yields than from gl *Aerobacter* strains will also produce 2,3-butanediol from man galactose, arabinose, isodulcite, adonitol, and glycerol.[illegible] bacter will ferment a much wider range of substrates than and, for this reason, may be useful for fermenting hydrol of fibrous materials which usually contain xylose. It has shown that wood hydrolysates can be successfully fermente this organism, producing a yield of 35% of 2,3-butanediol on the fermented sugar.[illegible]

MECHANISM OF THE BIOLOGICAL SYNTHESIS OF ACETOIN AND 2,3-BUTANEDIOL

Biological Synthesis of Acetoin

Pyruvic acid is readily fermented by 2,3-butanediol ba and is probably an important intermediate in the synthe acetoin and 2,3-butanediol from sugars. The synthesis of ac seems to be closely linked to the decarboxylation of pyruvic in fact, a model experiment, using light energy to decarbox pyruvic acid, will give higher yields of acetoin[illegible] than can b tained by irradiation of acetaldehyde.

The first clue to the mechanism of the biological synthesis of toin was the observation that acetylphenylcarbinol was formed ien benzaldehyde was added to a sugar solution being fermented yeast.[174,178,179] Since the compound obtained was optically active, was obviously a result of biological synthesis. Similar experinents, using acetaldehyde in place of benzaldehyde, have yielded tically active acetoin.[180] This suggested the presence of an zyme, called carboligase, capable of catalyzing the reaction:

$$2CH_3{\cdot}CHO \longrightarrow CH_3{\cdot}CHOH{\cdot}CO{\cdot}CH_3 \qquad (I)$$

The high yields obtained made it seem likely that some of e acetoin was formed from the sugar. Further experiment showed at this condensation occurred only in the presence of fermentg carbohydrates or pyruvate.[57,58,182] Acetoin can be formed from ruvate alone, although the yield may be increased by addition of etaldehyde.[181] Since the acetoin-forming enzyme has been found most workers to have the same properties as the decarboxylating zyme found in the same material, it is considered likely that ey are identical.[58,126,228,243] These conclusions have been quesoned by workers who have evidence that there are actually two zymes concerned since they obtained acetoin from acetaldehyde, thout simultaenous carbon dioxide formation.[122]

Since the formation of acetoin seems to require at least 1 mol "nascent acetaldehyde," formed from pyruvate by decarboxylaon, the reaction may be written:

$$CH_2CHO + CH_2{\cdot}CO{\cdot}COOH \longrightarrow CH_2{\cdot}CHOH{\cdot}CO{\cdot}CH_3 + CO_2 \qquad (II)$$

Acetoin-forming enzymes are widely distributed in nature, ing also found in yeasts of genera *Zygosaccharomyces* and *hizosaccharomyces*,[230] in bacteria,[157,158,228,251] in the seeds[243,244,252] d leaves [238] of higher plants, and in various animal tissues. pecially muscle.[77,232,271]

These acetoin-forming enzymes are probably not all identical, ce the optical activity of the acetoin obtained varies considerably, pending on the source of the enzyme preparation. Yeasts, bacia, and animal-tissue enzymes all give levo-rotatory acetoin, ile the enzymes of the higher plants give dextro-rotatory acen.[9,77,138,180,243,244,252,271] The acetoin produced by muscle tissue s $[\alpha]_D = -71°$ to $-81°$ in dilute aqueous solution as compared h $[\alpha]_D = +37°$ to $+10°$ for acetoin formed by leguminous seed meals. There must be at least two acetoin-producing enzymes, one found in plants and another found in animals and microorganisms. The highest rotations reported for dilute aqueous solutions of acetoin are $[\alpha]_D = -98°$ for acetoin formed by *Bacillus subtilis (B. natto)*,[250] and $[\alpha]_D = 103°$ for an enzyme preparation from yeast maceration juice.[58] *A. aerogenes* maceration juice also gives acetoin with a high *levo* rotation.[228] It is obvious that most enzyme preparations form both isomers of acetoin in unequal proportions or else they contain a racemase.[251] The specific rotation of optically pure acetoin is not known, so that it is not certain what proportions of isomers are produced by the various preparations.

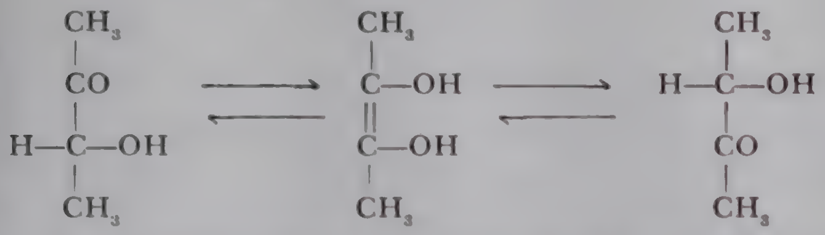
The enzymes of yeast and muscle, which have been studied the most extensively, are quite similar in their properties. They both carry out the general reaction:

$$CH_3CO{\bullet}COOH + RCHO \longrightarrow RCHOH{\bullet}CO{\bullet}CH_3 + CO_2 \qquad \text{(III)}$$

and it is possible to form a variety of compounds similar to acetoin. The muscle enzyme has the properties of a carboxylase. It requires diphosphothiamin and magnesium ions for coenzymes. It will decarboxylate other α-keto acids, such as α-ketobutyric and α-ketoglutaric acid, thus catalyzing reactions, such as:[15,77]

$$2C_2H_5{\bullet}CO{\bullet}COOH \longrightarrow C_2H_5{\bullet}CO{\bullet}CHOH{\bullet}C_2H_5 + 2CO_2 \qquad \text{(IV)}$$
$$C_2H_5{\bullet}CO{\bullet}COOH + CH_3CHO \longrightarrow C_2H_5CHOH{\bullet}CO{\bullet}CH_3 + CO_2 \qquad \text{(V)}$$
$$COOH{\bullet}CO{\bullet}CH_2{\bullet}CH_2{\bullet}COOH \longrightarrow CHO{\bullet}CH_2{\bullet}CH_2{\bullet}COOH + CO_2 \qquad \text{(VI)}$$

The compound formed in reaction III appears to derive the $CH_3CO{\bullet}$ group from the pyruvate, since addition of benzaldehyde to yeast fermentations[52,178,179] and propionaldehyde plus pyruvate to muscle preparation[15] gives as products the compounds $C_6H_5CHOH{\bullet}CO{\bullet}CH_3$ and $C_2H_5{\bullet}CHOH{\bullet}CO{\bullet}CH_3$, rather than the isomers containing a $CH_3CHOH{\bullet}$ group. By analogy, one would expect that acetoin formed by reaction (II) would derive the $CH_3CO{\bullet}$ end of the molecule from pyruvate and the ${\bullet}CHOH{\bullet}CH_3$ end from the acetaldehyde added. To check this point, heavy-carbon acetaldehyde was added to yeast juice containing pyruvate, the acetoin formed was isolated, and the position of the heavy carbon determined.[81] The isotopic carbon was found in both ends of the molecule, although there was a somewhat greater excess in the carbinol end, as expected. This means that the acetoin must have been partly formed through a symmetrical molecule or else racemization occurred after formation. As pointed out previously, yeast preparations often yield acetoin which is certainly not optically pure; furthermore racemization might occur during isolation, particularly if alkaline conditions are used.[244] Racemization probably occurs through the symmetrical dienol form whether catalyzed by alkali or enzymes.



$$\begin{array}{c} CH_3 \\ | \\ CO \\ | \\ H{-}C{-}OH \\ | \\ CH_3 \end{array} \rightleftharpoons \begin{array}{c} CH_3 \\ | \\ C{-}OH \\ \| \\ C{-}OH \\ | \\ CH_3 \end{array} \rightleftharpoons \begin{array}{c} CH_3 \\ | \\ H{-}C{-}OH \\ | \\ CO \\ | \\ CH_3 \end{array} \qquad \text{(VII)}$$

As a consequence of racemization, the labeled carbon will be found in both ends of the molecule. Experiments of this type should include measurements of the optical rotation, since samples of acetoin with the highest optical rotation would be expected to have the greatest concentration of isotopic carbon in the $CH_3CHOH\cdot$ end of the molecule. This type of experiment is rather important since another mechanism suggested for acetoin formation involves diacetyl as an intermediate:[147,242]

$$CH_3{\cdot}CO{\cdot}COOH \longrightarrow CH_2{=}C{=}O + CO_2 + 2H \qquad \text{(VIII)}$$

$$CH_2{=}C{=}O + CH_3CHO \longrightarrow$$
$$CH_3{\cdot}CO{\cdot}CO{\cdot}CH_3 \xrightarrow{+2H} CH_3{\cdot}CO{\cdot}CHOH{\cdot}CH_3 \qquad \text{(IX)}$$

If acetoin is formed by these reactions, rather than by reaction (II), the isotopic carbon should be equally distributed between both ends of the molecule.

The acetoin-forming enzymes of bacteria differ from those of yeast and animal tissues in not being able to utilize acetaldehyde. These enzymes form 2 mols of carbon dioxide and 1 mol of acetoin from 2 mols of pyruvate:

$$2CH_3{\cdot}CO{\cdot}COOH \longrightarrow CH_3{\cdot}CO{\cdot}CHOH{\cdot}CH_3 + 2CO_2 \qquad \text{(X)}$$

This has been found to hold for *B. subtilis*,[123,138] *A. aerogenes*,[13,228] and *Clostridium acetobutylicum*.[101] The enzyme system of *A. aerogenes* has been studied on cell-free preparations.[228] It differs from the yeast enzyme in being more sensitive to heat and alkalies and in not being active toward acetaldehyde in the presence of pyruvate. Although it is a carboxylase, using the same coenzyme, trapping experiments with bisulfite and dimedon failed to show intermediate formation of acetaldehyde which can be readily demonstrated with yeast carboxylase and even with bacteria growing on sugars.[177,230] The only products formed by decarboxylation of pyruvate were carbon dioxide and acetoin. Good yields of enzyme are obtained from cells grown in acid but not in alkaline media. Phosphate is necessary for the action of this enzyme and although no phosphorylated intermediates have yet been demonstrated, it is probable they exist.

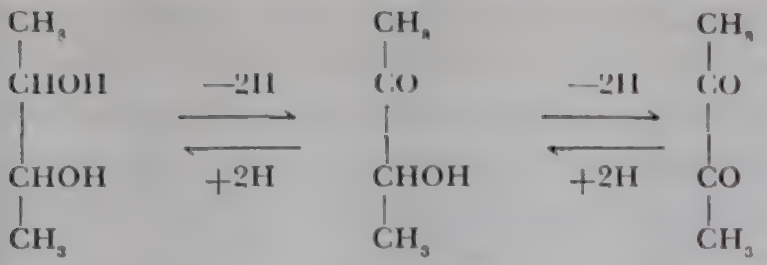
Very recently significant advances have been made in studies on the mechanism of acetoin formation. It was shown by Juni[101a] that α-acetolactic acid is an intermediate in the formation of acetoin from pyruvate by bacteria but apparently is not in yeast or mammalian tissue. These conclusions are supported by the results of other workers.[56a,59a,86a] An enzyme extract of *Aerobacter aerogenes* was resolved into components catalyzing the following reactions separately:

$$2CH_3COCOOH \longrightarrow CH_3COH\,(COCH_3)\,COOH + CO_2$$
$$CH_3COH\,(COCH_3)COOH \longrightarrow CH_3CHOHCOCH_3 + CO_2$$

The dextro-rotatory acetolactic acid formed is decarboxylated to levo-rotatory acetoin; the other isomer of acetolactic acid is not attacked at any appreciable rate. Yeast and mammalian tissue will not decarboxylate either isomer of this acid. However, tracer experiments indicate an asymmetric intermediate since the acetoin formed by pig-heart enzymes from pyruvate-2-C_{14} and acetaldehyde is labeled preponderantly in the carbonyl group, while that formed by yeast enzymes from acetaldehyde-1-C_{14} and pyruvate is labeled mainly in the carbinol group. There must be at least two mechanisms for the biosynthesis of acetoin and, in view of the known relation of acetoin to biological acetylation,[59] it would not be surprising if coenzyme A is involved in some systems.

Biological Synthesis of 2,3-Butanediol

2,3-Butanediol is probably formed by reduction of acetoin. Its more reduced state and the fact that some organisms form acetoin but no 2,3-butanediol,[7,8,9,35] both suggest that acetoin is formed before 2,3-butanediol rather than *vice versa*. These compounds are in equilibrium with each other and with diacetyl in fermentation solutions:



$$CH_3CHOHCHOHCH_3 \underset{+2H}{\overset{-2H}{\rightleftharpoons}} CH_3COCHOHCH_3 \underset{+2H}{\overset{-2H}{\rightleftharpoons}} CH_3COCOCH_3$$

(XI)

Under aerobic conditions, acetoin may be the major product of a 2,3-butanediol fermentation since all bacteria making 2,3-butanediol can oxidize it to acetoin under aerobic conditions.[111,135,191,259] Diacetyl is not usually formed in large amounts, but is present in strongly aerated cultures.[32,97] Acetoin is quite commonly formed in aerobic cultures. When the fermentation is vigorous, the redox potential falls and 2,3-butanediol is the main product, but when the sugar is used up, the redox potential rises and acetoin is formed.[230,263] Addition of a hydrogen acceptor, such as methylene blue, at a concentration of about 33 ppm, catalyzes the reaction and permits more effective use of air.[190] Removal of acetoin by oxime formation causes the reaction to go to completion.[191] Some 2,3-butanediol bacteria, such as *Aerobacter aerogenes,* will oxidize acetoin further, yielding such products as acetic acid if the aeration is prolonged.[233,278]

Diacetyl[176] is reduced by fermenting yeast, giving 2,3-butanediol with $[\alpha]_D = -2.4°$, while racemic acetoin[175] is reduced under the same condition to give 2,3-butanediol with $[\alpha]_D = -5.5°$. Since the 2,3-butanediol naturally formed from yeast[164] has a specific rotation of at least $-8.5°$, it is difficult to see how it could be formed by reduction of diacetyl, although it could be formed from acetoin, since the acetoin produced in a natural yeast fermentation is optically active. Bacteria will also reduce acetoin and diacetyl to 2,3-butanediol.[86]

Bacteria which oxidize 2,3-butanediol show stereochemical specificity and usually only oxidize an atom with either a D(−) or L(+) configuration, depending on the species. *Aerobacter aerogenes* and *Pseudomonas hydrophila* do not oxidize atoms with the D(−) configuration and thus will not oxidize the levo-rotatory (−,−) diol, but will rapidly oxidize the *meso* (+,−) diol. *B. polymyxa* can oxidize D(−) atoms and consequently will oxidize both the (−,−) and the (+,−) diols.[233] *Acetobacter suboxydans,*[74] *Acetobacter xylinum,* and *Acetobacter aceti*[80] also oxidize these diols specifically, all attacking the D(—) atom preferentially (see Chapter 1, Volume II).

The mechanism of the formation of 2,3-butanediol by microorganisms is not definitely established. However, from the work reviewed in the preceding pages, it appears likely that acetoin is first formed from pyruvate, or a closely related compound, and it is then reduced to 2,3-butanediol. Only one acetoin-forming enzyme may be present in bacteria since all those tested form (—) acetoin from pyruvate. Although acetoin has not been definitely isolated in an optically pure form, this may be due to enzymic racemization. However, it is necessary to postulate two acetoin reducing enzymes since, both (—,—) and (—,+) diols are commonly formed by bacteria. The simplest way to understand this is to postulate a 2,3-butanediol dehydrogenase reacting reversibly with D(—) atoms and another which acts reversibly with L(+) atoms. The formation of (+) acetoin by enzymes of higher plants makes it appear that at least four acetoin enzymes are found in nature; two which form acetoin and two which reduce it. However, it is possible that an enzyme exists which is capable of changing (—) acetoin into (+) acetoin, but which is incapable of synthesizing it from pyruvate. There is an interesting field here for further enzyme studies of fundamental importance in connection with the metabolism of pyruvic acid.

LABORATORY AND PILOT-PLANT STUDIES ON THE PRODUCTION OF 2,3-BUTANEDIOL

Many laboratory investigations have been conducted on the production of 2,3-butanediol from various substrates, some of which have been referred to previously. Several firms have carried out pilot-plant-scale fermentations for making of 2,3-butanediol, but few of their results have been recorded in the literature. Patent claims filed in Europe, Great Britain, the United States and Canada all give examples which indicate the use of pilot-plant equipment.

Fermentation Equipment

In order to obtain satisfactory results, butanediol fermentation must be carried out in modern, closed fermentation equipment. Owing to the sensitivity of most bacteria to traces of copper in the media, it is recommended to use only mild steel or stainless steel fermentors. The plant should be so designed that all pipes and vessels are readily sterilized by means of live steam under pressure. Valves and flange connections should be protected against contamination by steam pressure when not in use. Since all raw materials must be completely sterile, the use of a continuous high-pressure cooker[258] is desirable. If the proper alloy is used in the manufacture of the continuous cooker, it may also be employed for acid hydrolysis of grain mashes, such as was carried out by Joseph E. Seagram and Sons, Inc.[38,234,257] Installation of agitators in the fermentors is advisable for maintaining a constant and uniform temperature and to assist in proper aeration. Aerators should be mounted so that they can readily be removed for cleaning between fermentations as whole grain mashes have a tendency to plug them. Propagator equipment may be relatively small and must be so ar-

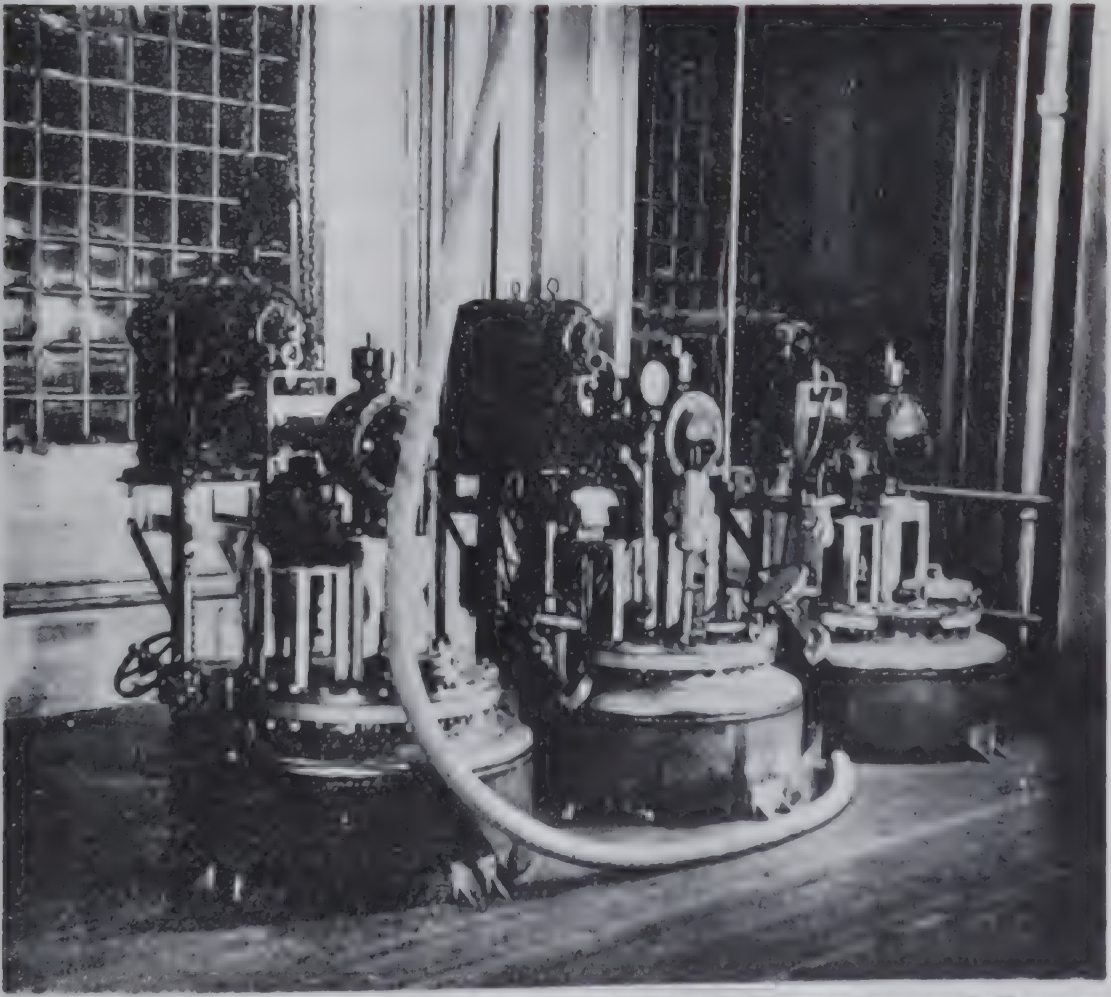


FIGURE 4. *Battery of 200-gal Stainless-Steel Experimental Fermentors in a Fermentation Pilot Plant* (Courtesy–Prairie Regional Laboratory, Saskatoon, Saskatchewan)

ranged as to eliminate possibilities of contamination during transfers. In general, much less inoculum is satisfactory in these fermentations than is necessary with yeasts in the production of industrial alcohol; inoculation with 0.1% by volume of active culture gives very satisfactory fermentations. A battery of three 200-gal stainless-steel fermentors in a pilot plant is shown in Figure 4.

Raw Materials

Most raw materials suitable for the production of industrial alcohol, as well as those containing pentose sugars, may be used for the manufacture of 2,3-butanediol. These may be classified into three main groups: (1) starchy substrates, including corn, wheat, barley, sweet potatoes, and potatoes; (2) sugars and sugar residues, such as beet and cane molasses; (3) wood hydrolyzates and pulp mill residues, including sulfite waste liquor. Bacteria used in the diol fermentation are much less tolerant of toxic substances than yeasts. Therefore, the use of very complex mixtures, such as sulfite waste liquor, or even cane or beet molasses, presents special problems. The most intensive studies to date have been made with whole ground cereal grains or degerminated corn and most pilot-plant data are based on the use of these materials. *Bacillus polymyxa* produces its own amylolytic enzymes which hydrolyze whole-grain mashes during fermentation. But if *Aerobacter aerogenes, Bacillus subtilis, Pseudomonas hydrophila,* or species of *Serratia* are used in the fermentation, the starch must first be converted to sugars. Conventional malting processes are not likely to be useful in saccharifying starchy substrates for these fermentations, since the introduction of contaminants is difficult to avoid. Acid hydrolysis has been employed most frequently, and studies have been carried out at Iowa State College using mold enzymes for the saccharification procedure.

Fermentation Processes

The 2,3-butanediol fermentations, with literature references to the substrates on which they were grown and conditions used, have been compiled[256] and are shown in Table 7. Relatively few of these publications refer to pilot-plant work, however.

TABLE 7. ORGANISMS PRODUCING 2,3-BUTANEDIOL

Organism	Substrate	Cultural condition	Literature references
Bacillus polymyxa	dextrose sucrose	anaerobic	5, 60, 61, 103, 210, 269
(*Aerobacillus polymyxa*)	xylose	anaerobic	5
	mannitol	anaerobic	5
	grain mashes	anaerobic	1, 2, 3, 4, 20, 22, 102, 120, 121, 129, 130, 131, 212, 232, 253, 266, 273
	grain mashes	aerobic	2, 22, 102, 131, 253, 273
	wheat starch	anaerobic	2, 69, 102, 103, 104
	cornstarch	anaerobic	120, 121
Aerobacter aerogenes including synonyms	dextrose	anaerobic	2, 4, 39, 89, 177, 186, 216, 263
A. faeni	dextrose	aerobic	2, 90, 120, 186
A. pectinovorum	xylose	anaerobic	29, 30, 213
	sucrose	anaerobic	29, 30, 70, 72, 73, 106, 213
	corn mash[a]	aerobic	90
	corn mash[b]	aerobic	120
	grain mash[c]	aerobic	234, 235, 236, 237
	starch[c]	aerobic	264
	starch[c]	anaerobic	264
	beet and cane molasses		72
	wood sugars[c]	aerobic	193
Aerobacter cloacae	dextrose	anaerobic	87, 214, 247
	xylose	anaerobic	213
	sucrose	anaerobic	213
Aeromonas hydrophila	dextrose	anaerobic	231
	xylose	anaerobic	231
Bacillus subtilis	dextrose	anaerobic	21, 165, 166
	dextrose	aerobic	21, 166
Serratia marcescens and other species	dextrose	anaerobic	168, 192
	dextrose	aerobic	168

[a] Mashes saccharified by fungus.
[b] Mashes saccharified by malt.
[c] Materials hydrolyzed by acid.

Source: Underkofler and Fulmer.[256]

FERMENTATIONS ON UNTREATED WHOLE GRAIN OR STARCH MASHES

Since whole grain mashes contain their own nitrogen and mineral sources with sufficient accessory nutrient factors to maintain a vigorous fermentation, their use has been intensively studied, employing *Bacillus polymyxa,* without preliminary acid hydrolysis or enzymic saccharification. Examples have been given[266] of an anaerobic fermentation, using *Bacillus polymyxa* in a wheat mash, a ground-corn mash, and a sweet-potato mash. Fermentation was carried out at 30°C, the mash was neutralized with 1% calcium carbonate, and fermentation was completed after 48 hours. The yields were approximately 10 lb of diol and 6 to 8 lb of alcohol for a 56-lb bushel of corn. With wheat, approximately 9.4 lb diol and 8.6 lb alcohol per 60-lb bushel were obtained. Schenley Distillers[143] at Lawrenceburg, Ind., conducted extensive pilot experiments on the fermentation of whole-grain mashes with *Bacillus polymyxa.* They carried out fermentations, using several strains of *Bacillus polymyxa,* obtained from the Northern Regional Research Laboratory, National Research Council of Canada, and others isolated in their own laboratory. In a 200-bushel per day capacity plant, they found that approximately 9 lb of 2,3-butanediol per bushel of wheat could be produced. They also used 10 to 12% corn mashes, obtaining an optimum yield of 10 lb per bushel with an average of approximately 9 lb per bushel of 2,3-butanediol. The National Research Council of Canada[273] recently published results of pilot-plant investigations on the *Bacillus polymyxa* fermentation. A yield of 8.9 lb of (—)-2,3-butanediol, and 5.9 lb of ethanol, per 60-lb bushel of wheat, was obtained, which is a fermentation efficiency of 90%. The best fermentations were obtained at 32°C, using a 15% mash buffered with calcium carbonate. Agitation increased the fermentation efficiency at 96 hours, but decreased the diol to ethanol ratio. Reduced pressure did not change the efficiency but markedly reduced the ratio of diol to ethanol. Aeration had little effect in the large fermentors. In these pilot-plant experiments, it was found that adding inoculum at levels of 0.2, 0.1 and 0.05% gave completely satisfactory results. The use of this low amount of inoculum is of importance in plant design, as it eliminates large propagator equipment. On the above basis, only 10 to 20 gal of inoculum would be required for a 20,000 gal fermentor. In these pilot-plant studies, the inoculum was prepared from agar slants by three 24-hour stages. Transfers were made from slant to 10 ml of medium. This was employed to inoculate 200 ml of medium and the 200 ml culture to inoculate 2,000 ml, which was finally used to inoculate the 600 gal fermentation. The inoculum medium consisted of 1% ground wheat, 3% starch, 1.5% yeast extract and 1% calcium carbonate. On this medium, 500 million organisms per milliliter, as estimated by direct count with a

Petroff-Hauser plate, were obtained after an incubation period of 24 hours. Malt sprouts or shorts offer a good commercial substitute for yeast extract as the added nutrient. Where no nutrient is added, growth is slower and the numbers are much lower at 24 hours. The calcium carbonate may be omitted if the incubation period is not longer than 24 hours. Because the effect of aeration in large fermentors is less marked than on a laboratory scale, it was concluded that the beneficial effects of aeration in commercial practice would not justify the initial cost of installing air compressors and aeration equipment. This also applies to the application of reduced pressure. The installation of agitators would be advisable to facilitate removing the mash and for maintaining a constant and uniform temperature. If the cooked mash were to be cooled in the fermentors, agitation would also be necessary.

Barley mashes were also successfully fermented by *Bacillus polymyxa* on the pilot-plant scale.[253] It was found possible to ferment mashes up to 14.5% of concentration. The highest efficiency, 94%, was obtained with a 14% mash. The yield from the 48 lb bushel of barley was 6.9 lb of diol and 5 lb of alcohol. Comparison of the product yields of wheat and barley fermentations are given in Table 8. Estimates showed that on the basis of the long-term average grain prices in western Canada, a 10% cost reduction could be expected if barley, rather than wheat, was used as a raw material for this fermentation.

TABLE 8. YIELDS FROM WHEAT AND BARLEY FERMENTATIONS[a]

Products	Wheat	Barley
Butanediol recovered, gal	846	781
Alcohol recovered, gal 95%	675	610
Bran or hulls, 13.5% moisture, tons	1.89	5.12
Residue, 13.5% moisture, tons	12.20	9.53
Total residues, tons	14.09	14.65

[a] Basis: 30 tons a day—1,000 bu wheat or 1,250 bu barley at 13.5% moisture.
Source: Tomkins, Scott, and Simpson.[253]

While whole-grain mashes do not present any serious fermentation problems and have certain advantages in that they contain growth factors, nitrogen, and minerals, the large percentage of nonfermentable matter complicates subsequent diol-recovery operations. Furthermore, the nonstarch portions of the grain m be seriously degraded as a feed by the recovery treatments. F these reasons, many laboratories carried out experimental wo on purified corn or wheat starch and also on granular wheat flo or "alcomeal," used for industrial-alcohol production. The labor tory investigations of additional nutrient requirements to obta good fermentations of purified starch by *Bacillus polymyxa* ha already been mentioned. Apparently no pilot-plant studies ha been made with purified starch, although laboratory studies have dicated that cornstarch could be fermented satisfactorily wi *B. polymyxa.* The best medium of Kooi, Fulmer, and Unde kofler[131] contained 7.5 g commercial cornstarch, 0.5 g corn glute 0.006 g potassium permanganate and 0.5 g calcium carbonate p 100 ml. Yields from 200 ml fermentations on this medium we 26.8% diol and 14.8% ethanol, based on the weight of starch presen The efficiency of this fermentation was slightly lower than th best fermentations which may be obtained on whole grains. Ho ever, the total solids were reduced by 50%, resulting in greater ea of filtration.

ACID-HYDROLYZED WHOLE-GRAIN OR PURIFIED-STARCH MASHES

In 1942 and 1943 quite extensive studies were made on the ac hydrolysis of whole grains for the 2,3-butanediol fermentation pr duced by *Aerobacter aerogenes.* A number of industrial firms, t gether with the Northern Regional Research Laboratory, th University of Wisconsin, and the University of Alberta in Canad carried out laboratory and in some cases pilot-plant studie Descriptions of the pilot-plant work, conducted by Joseph E. Se gram and Sons, Inc., have been published.[55,234,235,236] Many facto affecting the diol yields, mash preparations, bacterial culture nitrogen supplements, pH, temperature, aeration, sugar conce trations, and time of fermentation were investigated in laborato studies and most of these confirmed by later pilot-plant work o corn and wheat mashes. The grain mash was acid hydrolyzed b a continuous process developed by the same firm.[237] Two differe cultures were compared for their fermentation ability, No. 1 obtained from the Northern Regional Research Laboratory an No. 766 from the University of Wisconsin. The second gave high yields and was not as sensitive to many factors as culture 199. I Table 9, the fermentation yields obtained with culture 766 on number of pilot-plant runs are shown.

TABLE 9. PILOT-PLANT FERMENTATION YIELDS OBTAINED WITH CULTURE 766[a]

	Fermentor 1		Fermentor 2	
Run No.	Diol, lb per bu	Ethanol, lb per bu	Diol, lb per bu	Ethanol, lb per bu
91	13.1	2.69	13.8	1.51
92	13.3	2.69	13.1	1.51
93	14.9	1.56	13.4	0.57
94	13.0	2.39	13.1	1.05
95	14.2	2.35	15.7	1.24

[a] Fermentation medium: Acid-hydrolyzed wheat mash.
Source: Stark, Adams, Kolachov, and Willkie.[225]

The grain mash was acid hydrolyzed, neutralized to pH 6, nd 0.5% calcium carbonate and 0.2% urea were added. Fermentaions were conducted at 86 to 90°F, with agitation, and aerated at he rate of 5 cu ft per minute per 1,000 gal of mash. The average ilot-plant yields, as shown in Table 9 were approximately 14.2 lb f butanediol per bushel of grain; the maximum yield obtained as 15.7 lb. Small quantities of ethanol (approximately 0.75 to .5 lb per bushel) and also traces of acetoin (0.6 to 1.2 lb per bushel) vere produced. The Seagram investigators concluded that a practial fermentation for the production of 2,3-butanediol from grain nash could be developed and that additional work ought to raise he yield to 15 or 16 lb per bushel. Wheat and corn were satisfacory raw materials.

The production of 2,3-butanediol by *Aerobacter aerogenes*[200] as been studied on a large laboratory scale, using apparatus deigned for the acid hydrolysis of wheat mashes with agitation and nother type of apparatus for hydrolysis without agitation.[186] This vork was carried out in glass vessels. It was found that the stirring f wheat mashes reduced the time necessary for hydrolysis to one hird that required with unstirred mashes. Yields on acidydrolyzed wheat were approximately 28 g of diol per 100 g of rain.

The Northern Regional Research Laboratory carried out work n the fermentation of acid-hydrolyzed whole grain (corn and vheat) mashes and also of acid-hydrolyzed purified corn and wheat tarch.[264] The results on the last are the only ones which have so ar been published, although it is mentioned that acid-hydrolyzed vhole-grain mashes were found to yield 13 to 14 lb of butanediol er bushel of grain processed. From work with whole grain mashes he metallic ion content was not an important factor in determining the outcome of the fermentation. It was found, however, w purified starches were used, that certain heavy metals were This is probably due to the lack of proteins in the mash w would protect the organism against such substances. Trac some metals play an important role in the production of butanediol from acid-hydrolyzed starch. The presence of c ions, particularly those of manganese, cobalt, or molybdenum reflected in a decreased production of diol and an increased pr tion of ethanol under aerobic conditions and they also caus increased rate of fermentation under anaerobic conditions. balance between these trace elements seems to be the impo factor. It was possible to treat the mashes either by an ion-exc agent to remove certain of these elements and to bring them balance or by the addition of traces of other metallic ions, as copper and zinc. The second method is only applicable amounts of trace elements present in the starch are known so they can be balanced. It is somewhat easier to treat the s hydrolysate with an ion-exchange column and remove certain Acid-hydrolyzed starches fermented by *Aerobacter aerogenes* given 13.5 to 18 lb of 2,3-butanediol per 34 lb of pure s (equivalent of 1 bushel corn).[229] It has been found in start fermentations that all mashes produced large quantities of eth the ratio butanediol to ethanol being approximately 2:1, i.e., similar to that obtained with *Bacillus polymyxa* under the conditions. With proper aeration, however, the yield of eth was reduced and much higher ratios were obtained. The of various growth factors, such as biotin, nicotinic acid, par benzoic acid, pyridoxin, inositol, thiamin, riboflavin, and ca pantothenate were studied. No difference in the proportio ethanol and diol were observed when aerated glucose cultures supplied. Each liter of the saccharified starch medium cont 0.25 g $MgSO_4 \cdot 7H_2O$, 0.08 g KH_2PO_4, 2.0 g urea, and 5 to $CaCO_3$. The first two salts were added to the medium before ing and the urea and calcium carbonate were each ster separately and added before inoculation. The quantity of cal carbonate employed was sufficient to neutralize at pH 5.5 to 6 also to act as a means of pH control during the fermentation. 8 l lot of medium was inoculated with 100 ml of an 8- to 20 inoculum culture. The fermentations were conducted in ing aluminum drum fermentors, which were operated at

to 12 rpm, 5 psi gage pressure, and an air flow of 50 to 100 ml
r minute which had been found near optimal in previous studies
diol production from glucose.

ZYME-HYDROLYZED WHOLE-WHEAT AND STARCH MASHES

Although many investigators undoubtedly have used malt to
drolyze starch for butanediol fermentations, relatively few reports
ve been published on this process. Possibly this is due to the
t that use of the conventional malting process necessarily intro-
ces many other organisms which interfere with the subsequent
ol fermentation. If the partially converted mash is autoclaved
ior to fermentation, extra equipment, steam, and water is re-
ired and there is a 20% reduction in yield as well.[236] The pro-
iction of 2,3-butanediol by *Aerobacter* from corn has been studied,
ploying malt saccharification to determine whether brewery
uipment and technique could be adapted to the fermentation.[illegible]
ie ground corn was mashed with water, cooked, and saccharified
means of barley malt. The solids were removed by filtration
obtain a clear wort which contained the equivalent in reducing
gars of about 90% of the starch present in the corn and the
alt employed. Urea and calcium carbonate slurries were sterilized
parately and added to the wort before fermentation. These ex-
riments showed that in a wort containing up to 12% reducing
gar as maltose, fermentation was satisfactory, but with higher
gar concentrations decreased yields were obtained. It was neces-
ry to use aeration for rapid fermentation and yields of the diol
tained were about 20% of the weight of maltose present in the
ort. By the addition of freshly prepared malt extract to the
edium after an active fermentation had started, it was possible
obtain better fermentations with yields as high as 38% of the
ight of the initial maltose in the wort. In pilot-plant fermenta-
ns, at least 35% was obtained.

In work carried out in the Department of Soils at the University
Alberta, in 1943, malt was used to hydrolyze whole wheat and
rch mashes.[19] Low sugar concentrations were used in most of
experimental work and excellent fermentations were obtained
a number of cases. However, the use of a malt-hydrolyzed
dium did not appear to be feasible in larger-scale work, owing to
difficulty of obtaining high sugar concentrations and good sugar
lization by the organism.

In an investigation on starch hydrolysis by *Endomycopsis fibuliger* at the Northern Regional Research Laboratory,[275] mixed cultures of this yeast and *Aerobacter aerogenes* were used for the production of 2,3-butanediol from 15% wheat mash. A tube, containing 100 ml of 15% wheat mash, was inoculated with 2 ml of the cell suspension of a strain of *E. fibuliger* and simultaneously with 5 ml of a 20-hour culture of *A. aerogenes,* grown in a 5% malt-converted wheat mash. Before inoculation, each tube received 1 ml of sterile 20% urea solution to provide the necessary nitrogen for *A. aerogenes.* All cultures were incubated at 38°C for 72 hours. Two cultures received 10 ml of air per minute for 72 hours and the other two cultures were not aerated. It was found that the fermentation in unaerated cultures was much better than usual. Apparently, the presence of the yeast, or the liberation of some nutrient factor, stimulated the development and action of *Aerobacter,* especially in regard to the production of ethanol. The best yields obtained were 27% of 2,3-butanediol and 13% of ethanol, based on the weight of the starch present.

Corn mashes can be fermented satisfactorily by *A. aerogenes,* following saccharification by *Aspergillus oryzae.*[90] From a medium containing 15% corn meal, 0.4% dipotassium phosphate, and 1% calcium carbonate, up to 38% of 2,3-butanediol was obtained, based on the weight of the starch present. To date, no work has been reported with enzymes produced by submerged cultures of *Aspergillus niger* for saccharification of mashes prior to fermentation with any of the butanediol-producing bacteria. Investigations are now under way in the Prairie Regional Laboratory at Saskatoon, Saskatchewan, to determine the usefulness of these enzymes in the *Bacillus subtilis* fermentation for 2,3-butanediol and glycerol production.

STUDIES ON PURIFIED SUGAR AND BEET AND CANE MOLASSES

Laboratory investigations at many centers have shown that high yields of butanediol can be obtained with aerated cultures of *Aerobacter aerogenes* grown on dextrose solutions. Sugar solutions up to 10% concentration can be fermented in less than 48 hours with a yield of 80 millimols of glycol per 100 millimols of glucose under optimum conditions.[186] If anaerobic cultures of this organism are used, the yield is approximately half this amount. No pilot-plant experiments on the fermentation of dextrose have been reported.

though undoubtedly diol was produced for recovery and chemical studies by this method. Several laboratory studies have been made on the fermentation of sucrose, largely as preliminary work to the utilization of beet or cane molasses. The most detailed studies reported appear to be those from the Imperial Chemical Industries in Britain.[70,71] Detailed investigations were made on the conversion of sucrose to 2,3-butanediol with *A. aerogenes,* investigating pH, sucrose concentration, optimal temperatures, aeration, and the addition of organic nutrients. The pH optimum range for diol production was found to be 5 to 5.5 and the optimum temperature 35°C. Rapid fermentation and high diol yields were obtained with an initial sucrose concentration in the range of 5 to 20%. A maximum yield of 87.4% of theory was obtained with a sucrose concentration of 15%. In aerated, stirred cultures, containing 10% sucrose with 1.2% calcium carbonate, rapid fermentation began after a lag period of 5 to 7 hours, and was complete in 24 hours. It was found that with unaerated cultures, high yields of ethanol, amounting to 12 to 14% of the sugar fermented, and of lactic acid (5 to 10%) were obtained. However, under optimum conditions approximately 85% of theory of 2,3-butanediol and relatively small concentrations of ethanol were obtained. Different types of molasses were investigated, including high-test molasses, crude-beet molasses, South African blackstrap molasses, West Indian blackstrap molasses, home-grown beet molasses, and crude commercial glucose, manufactured from corn. A rapid, complete fermentation of blackstrap molasses took place under suitable conditions, leading to 75 to 80% of theoretical yield of diol. Rates of fermentation compared unfavorably with purer substrates, such as sucrose or high-test molasses, but by using acclimatized strains of the organism, both fermentation time and yields could be markedly improved. Fermentations on a 12.5 l scale were carried out successfully and there is good reason to expect that this fermentation can be put into large-scale practice. In addition to the diol, considerable amounts of ethanol, lactic acid, and acetoin were formed under certain conditions. The relative amounts of these products, particularly the diol to ethanol ratios, depended on aeration and on protein and trace-metal concentrations of the medium.

The National Research Council of Canada has recently carried out pilot-plant studies on the production of 2,3-butanediol from Ontario sugarbeet molasses. Some twelve or fifteen 800-gal fermentations have now been run, with automatic pH control in most of them. It was found that the addition of phosphate increased the rate and, in common with all rate increasing factors, decreased the diol to alcohol ratio. By decreasing the amount of phosphate and increasing the aeration rate, it was possible to get a ratio greater than 3:1 and still complete the fermentation in less than 72 hours. Many of these fermentations were completed within 36 to 48 hours, 95 to 98% of the sugar having been utilized with a total fermentation efficiency of 90 to 95%. It is apparent that there would be little difficulty in obtaining a commercially feasible 2,3-butanediol molasses fermentation using *Aerobacter aerogenes.*

If it is desirable to produce *levo*-2,3-butanediol, it will be necessary to use *Bacillus polymyxa.* So far only preliminary pilot-plant work has been carried out with this organism on Ontario beet molasses, but fermentation of pure sucrose under controlled pH conditions has been studied recently.[269] It was found that the use of 8% sucrose gave the most efficient fermentation in 30 hours at pH 6, yielding 65 millimols of diol per 100 millimols of invert sugar fermented. This laboratory investigation has been discussed before. Ammonium hydroxide was found to be much superior to either sodium or potassium hydroxide as neutralizing agent, because of its effect on the rate of sugar dissimilation.

Recently, Simpson and Stranks[228a] have reported a laboratory study on some factors affecting the fermentation of beet molasses by *B. polymyxa.* Strains selected on the basis of their ability to ferment glucose to 2,3-butanediol gave better diol yields from beet molasses when acclimatized to beet molasses medium. Best yields were obtained by adjusting the molasses media to pH 5.6 prior to sterilization since this gave the optimum pH of 6.2, after autoclaving at 15 psi gage pressure for 15 minutes. Most rapid fermentation and best diol yields were obtained by agitation and aeration of the medium. Addition of organic stimulants to molasses medium resulted in more rapid fermentations and increased final diol yields in several cases. Yeast extract, malt sprouts, whole wheat and wheat bran were the most effective. Wheat bran was adopted as the cheapest adjuvant, and was tested at several levels. With the molasses sample employed, fermentation of 10% molasses medium containing 0.11% wheat bran at 35°C, with agitation and aeration, was complete in 60 to 72 hours and yielded 2.01 g diol plus 0.88 g ethanol per 100 ml of medium.

Subsequently Murphy, Stranks, and Harmsen[154b] reported that *B. polymyxa, A. aerogenes, B. subtilis,* and *Serratia marcescens,* when cultivated on molasses media, were stimulated in growth and butanediol production by adding organic substances, such as wheat bran, yeast extract or corn-steep liquor. It was found that the principal beneficial factor in all these substances was the phosphorus content. Beet molasses was found to be an excellent substrate for butanediol production by all these organisms when sufficient soluble phosphate salts were added. It is necessary to add the phosphorus as orthophosphate, or in a form which readily yields orthophosphate. However, the natural organic substances contained, in addition to phosphate, some minor unidentified factors. Addition of wheat bran to a molasses medium containing added phosphate resulted in a small but noticeable improvement in 2,3-butanediol yield when fermented by *B. polymyxa.*

Other studies have also been made in the Canadian Research Council on the fermentation of molasses with *Bacillus subtilis* and *Pseudomonas hydrophila* for making butanediol. These investigations have not yet been carried out on the pilot-plant scale, but there is every reason to expect that most of the butanediol-producing bacteria can be satisfactorily acclimatized to the utilization of beet and cane molasses.

Most recently, Murphy, Watson, Muirhead, and Barnwell[154c] have reported extensive study of beet-molasses fermentation by two strains of *Pseudomonas hydrophila.* These strains differed from those employed by Stanier and Adams[231] in that they produced 2,3-butanediol containing a high percentage of racemic mixture, gave higher yields of diol, and fermented sugars more rapidly and completely. Dissimilation of molasses sugars was practically complete in 24-hour fermentations in molasses media containing 5 and 7.5% sugar, and much slower and incomplete when 10% sugar was present. The media contained, in addition to molasses, ammonium phosphate and wheat bran to supply minor nutrients. Yields of products from numerous fermentations, conducted with agitation and aeration at 32°C, and without pH control, averaged 87.8% of theoretical diol plus acetoin, 13.1% ethanol, and 2.2% organic acids. Fermentations by high-yielding strains of *A. aerogenes* on media containing the same molasses averaged 82.9% of theoretical diol plus acetoin, 19.8% ethanol and 4.5% organic acids. Aeration had less tendency to convert diol to acetoin in the *P. hydrophila* fermentation. It, therefore, appears that *P. hydrophila* produces a higher yield of diol plus acetoin than *A. aerogenes*. In two fermentations, with the initial pH adjusted to 6.2, approximately 95% of theoretical diol and no ethanol were produced. The mixture of stereoisomeric 2,3-butanediols produced by *P. hydrophila* by physical methods was calculated to contain 50% *racemic*, 48% *meso* and 2% *levo*.

FERMENTATIONS ON HYDROLYZATES OF WOOD OR AGRICULTURAL RESIDUES

Since the 2,3-butanediol-producing bacteria ferment xylose and mannitol[119,136,139,144] as well as glucose, fructose, and sucrose, the sugars derived from different cellulosic materials, such as wood waste and agricultural residues, ought to be considered as possible raw materials. Workers in the Northern Regional Research Laboratory[63] have used *Aerobacter aerogenes* to ferment saccharified agricultural residues, such as corncobs, bagasse, oat hulls, and flax shives, but no details of these experiments have as yet been published.

Hydrolyzates of both hard and soft woods, including southern red oak, Douglas fir, white spruce, and southern yellow pine, have been used as substrates for the production of 2,3-butanediol.[193] Two types of treatment of the wood sugars, prior to fermentation, were used. In one, the solutions were neutralized to pH 6 by addition of a slurry of lime or alkali, after which the precipitated calcium sulfate was removed by filtration. The second method involved the addition of excess alkali up to pH 10 or 11, filtration, and then readjustment with sulfuric acid to pH 6 and filtering. Dilute solutions from the first treatment fermented well, but addition of malt sprouts or yeast extract was necessary on more concentrated solutions. The second method of treatment gave good fermentations in solutions containing up to 17.8% sugars. Approximately 35% of the fermented sugar was converted to 2,3-butanediol. It was possible to acclimatize the organism to wood-hydrolyzate media by repeated transfers. With acclimatized cultures, improved yields were obtained on wood-hydrolyzate media, without addition of auxiliary substances or treatment involving more than direct adjustment of the pH to 6.0.

RECENT FERMENTATIONS

Since this chapter was first written, a number of papers have appeared confirming and extending previous work on the production of 2,3-butanediol from various substrates. These include work on the fermentation of waste sulfite liquor,[154a] cellulosic materials,[58a] and wheat,[48a] as well as further work on the fermentation of glucose by *Aerobacter aerogenes*[187a] and *Bacillus mesentericus*.[9a]

CONTAMINANTS AFFECTING 2,3-BUTANEDIOL FERMENTATIONS

Bacteriophage and foreign spore-forming bacteria are the most important contaminants likely to affect the 2,3-butanediol fermentation. The most frequent cause of contamination in pilot-plant fermentations of wheat arises from insufficient sterilization of the whole-wheat mash, rather than from the entrance of foreign organisms into the system after sterilization.[21] Most of the isolated contaminating organisms were spore formers. It has been found that No. 1 Manitoba Northern Wheat has the lowest bacterial count, but in lower grades, counts ranged from 280,000 to 164,000,000 for bacteria, 420 to 1,870 for fungi, and 6,200 to 64,000 for yeasts per gram of grain.[99] Experiments on the use of ultraviolet germicidal lamps made it possible to reduce cooking time by 15 minutes, but recontamination makes this process unlikely to be of much commercial value. The effect of bacteriophage has been studied in relation to diol production,[105] but no relationship has been found between the ability of strains of *B. polymyxa* to produce diol and their susceptibility to bacteriophage. It was found that bacteriophage could be carried through the spores of *B. polymyxa* and that it was quite heat resistant, since a temperature of 80°C for 60 minutes did not inactivate it.

Workers at Joseph E. Seagram and Sons, Inc., have also investigated the bacteriophage present in *Aerobacter aerogenes* fermentations. Standard methods of phage isolation and culture failed to demonstrate the presence of phage conclusively, despite the fact that positive indications of this type of infection were obtained. It was found that the addition of mash and calcium carbonate to the tubes cleared by phage activity of the original filtrate before filtering enabled active cultures of the phage to be obtained.

RECOVERY PROCESSES AND EQUIPMENT

Because of its high boiling point, 2,3-butanediol is not easily recovered from fermented mashes. The initial steps in all suggested recovery methods are similar and consist of removal of ethanol, filtration or screening, and concentration of the liquor by evaporation.[241] Ethanol, which is a by-product of the fermentation, can be recovered either by a separate operation or during evaporation. Methods which have been suggested for the recovery of 2,3-butanediol from evaporator sirup are: spray drying,[143] drum drying,[127] chemical conversion,[78,225] dialysis,[47] solvent extraction,[94,127,137] ethanol precipitation,[34] kerosene distillation,[127] and steam stripping.[113,246] Only those considered most promising will be described here.

Spray Drying

A recovery process for fermentation residues, containing high-boiling constituents, such as glycerol, butylene glycols, ethylene and propylene glycols, was patented[51] in 1942. The method consists of spraying the fermentation residue into a stream of inert gas at a temperature of approximately 900°F, to provide vaporized volatile products and separating the solids from the gas stream while the temperature is above the point at which condensation of volatile products can occur, then cooling the stream, and condensing the products to be recovered. In spray-drying of 2,3-butanediol the beer is concentrated by evaporation to about one fourth to one fifth its original volume and the heated concentrate is flashed into a vacuum chamber.[143] The vapors consisting of steam and diol are directly fractionated and the diol collected separately. The residual solids from this process are used as animal feed.

Chemical Conversion

A chemical process for the recovery of 2,3-butanediol from fermentation beers has been described.[225] The method consisted of distilling an acidified mixture of whole beer and formaldehyde into a decanter which permitted return of the lower aqueous layer to the distilling vessel. The top oil layer, which was collected, contained, as the formal, 98 to 100% of the butanediol present in the beer. The 2,3-butanediol formal was then resolved into two geometrical isomers. The formal was allowed to react with acid methanol and converted to 2,3-butanediol and methylal in 95% conversion. A method was developed for the recovery of formaldehyde and methanol from the methylal. A similar process, using butyraldehyde as the solvent, has recently been developed by the National Research Council of Canada.[248] In this process, butyral is formed, which is then separated by distillation, and later 2,3-butanediol can be recovered by acid treatment.

Solvent Extraction

In the laboratory, 2,3-butanediol can be readily recovered from fermentation beers by continuous extraction with diethyl ether.[18,69,100,118,159] Extraction by means of *n*-butyl alcohol has been employed in several pilot-plant installations. Several ternary liquid systems have been investigated, including diol, water, and individual solvents which might be suitable, such as butyl alcohol, butyl acetate, butylene glycol diacetate, and methylvinylcarbinol acetate.[188] This experimental work allowed the investigation of respective advantages of the solvents and the desirable operating temperatures.

Steam Stripping

Possibly, the most practical method of recovering 2,3-butanediol from fermentation sirups is through vapor-phase steam extraction. This method has been successfully employed on pilot-plant scale by both the Northern Regional Research Laboratory and the National Research Council of Canada. A process for the recovery of *meso*-2,3-butanediol has been described in which the concentrated liquor is steam stripped in a packed column at elevated temperatures.[25] A similar process has been successful, using a whole-wheat mash fermented by *Bacillus polymyxa*.[273] The mash was stripped of ethanol, screened and concentrated, and the resulting sirup was then passed through a steam-stripping column and the *levo*-2,3-butanediol in the concentrate was recovered by rectification. The composition of intermediate products, performance of pilot-plant equipment, and data and recommendations for the design of a large-scale plant were worked out.

RESIDUES AND WASTES

It is unlikely that the fermentation of whole-grain mashes for the production of 2,3-butanediol will be able to compete with a molasses fermentation, unless the residues can be recovered in the form of a high-grade protein feed. Very little experimental work has been carried out to determine whether the organisms employed in the fermentation produce sufficient riboflavin or other vitamins to enhance the feeding qualities of the nonfermentable portions of the grain. Analysis of the residues obtained from fermentation of whole-wheat mashes by *Bacillus polymyxa* in the National Research Council of Canada[273] are shown in Table 10, with data for other fermentation residues given for comparison. The ash content of of the stripper residue was high, because it contained all the calcium carbonate added to the mash. The butanediol content of these residues is higher than necessary, since it could be reduced by more efficient stripping of the stripper residue or by more complete washing of the bran prior to drying. No thiamin was present in any of the solids, but the riboflavin content of the stripper residue represents approximately 100%, and the nicotinic acid content

TABLE 10. ANALYSES OF PILOT-PLANT BUTANEDIOL WHEAT MASH AND COMMERCIAL RESIDUES

	Dried stripper residue		Dried screen solids	Distillers' grains from rye	Brewers' grains over 25% protein	Strained distillery slop[a]
	No. 1	No. 2				
Butanediol, %	1.27	1.58	0.53			
Moisture, %	8.42	11.8	10.7	7.2	7.5	10.0
Soluble solids, %	59.4	55.6	0.0			
Ash, %	11.7	13.5	1.65	3.9	3.5	6.6
Total nitrogen, %	5.59	6.02	1.21	3.7	4.24	4.91
Water-soluble nitrogen, %	4.05	4.40	0.21			
Total P_2O_5, %	2.33	2.96	1.33	0.83	0.99	
Available P_2O_5, %	2.21	2.82	1.13			
Total K_2O, %	0.11	0.10	0.07	0.24	0.09	
Total CaO, %	5.4	5.5	0.74		0.16	
Crude protein, % N × 6.25	34.0	37.6	7.56	23.1	26.5	30.7
Fat, %	5.1	5.0	3.4	7.8	6.9	15.4
Fiber, %	2.0	2.3	27.0	10.9	14.6	4.4
Nitrogen-free extract, %	37.9	29.8	49.5	47.1	41.0	32.9
Thiamine, ppm	0.0		0.0			
Riboflavin, ppm	1.64		1.3			
Nicotinic acid, ppm	88		6.4			

[a] Calculated on a 10% moisture basis.

about 50%, of that present in the wheat, although the residue was only 30% of the original weight of wheat used. The bran contained about 50% of the riboflavin and 100% of the nicotinic acid usually present in untreated wheat brans.

The stripper residue appears to have possibilities as a livestock feed, though feeding tests were not carried out. It is higher in protein and minerals than other commercial residues with which comparsons were made, but somewhat lower in fat and fiber content. The dried bran would be of little value as a feed other than roughage. Neither the stripper residue nor the bran would be useful as a fertilizer without being enriched with phosphorus or potash.

COST OF PRODUCTION

Because none of the 2,3-butanediol fermentations has ever been applied on an industrial scale, very little detailed information is available on which to base costs of production. An estimate, based on pilot-plant operations by Schenley Distillers,[143] indicates that butanediol may be produced at a price of 10 to 12¢ per pound. Calculations from the Northern Regional Research Lavoratory,[127] based on grain at 70¢ per bushel and a diol yield of 9.86 lb per bushel in an 8,500 bushels per day plant gave a cost of 10.62¢ per pound. Both estimates are appreciably lower than those based on pilot-plant studies of the *Bacillus polymyxa* fermentation carried out in the national research Council of Canada.[253,273] An analysis of costs and credit distribution using wheat is shown in Table 11

TABLE 11. COST AND CREDIT DISTRIBUTION FOR BUTANEDIOL PRODUCTION

Distribution	Percentage of total	Cents per imperial gal
Costs		
Fixed costs	14.30	39.10
Wheat	47.70	130.10
Other raw materials	5.46	14.89
Steam	8.15	22.20
Wages	13.00	35.50
Other production costs	11.39	31.13
	100.00	272.83
Credits		
Butanediol	63.8	173.15
Ethanol	17.2	47.88
Dried solids	19.0	51.80
	100.00	272.83

Source: Wheat, Leslie, Tomkins, Milton, Scott, and Ledingham.[273]

and a comparison of costs of wheat and barley fermentations in Table 12. With present prices of cereals, cost of production from grains is unduly high and there is little prospect of commercial development until cheaper sources of raw materials become available. Undoubtedly *(meso-dextro)*-2,3-butanediol could be produced by *Aerobacter aerogenes* on molasses for considerably less per pound but no cost analyses have been published.

TABLE 12. COSTS FOR WHEAT AND BARLEY FERMENTATIONS[a]

Production costs and credits	Wheat		Barley	
	Present	Long-term average	Present	Long-term average
Costs	dollars	dollars	dollars	dollars
Grain[b]	1,270.00	870.00	1,025.00	637.50
Utilities (steam, power, water)	265.00	265.00	261.00	261.00
Other costs (wages, fixed costs, etc.)	941.50	941.50	941.50	941.50
Total daily cost	2,476.50	2,076.50	2,227.50	1,840.50
Credits	dollars	dollars	dollars	dollars
Ethanol at $0.60 per gal	405.00	405.00	366.00	366.00
Residues at $30.00 per ton	422.70	422.70	439.50	439.50
Butanediol	1,648.80	1,248.80	1,422.00	1,035.00
	cents	cents	cents	cents
Cost butanediol per lb	19.5	14.8	18.2	13.3

[a] Basis: 30 tons a day—1,000 bu wheat or 1,250 bu barley at 13.5% moisture.
[b] Wheat—present price, $1.27 per bu; long-term average, $0.87 per bu.
Barley—present price, $0.82 per bu; long-term average, $0.51 per bu.
Source: Tomkins, Scott, and Simpson.[253]

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PART III. THE PRODUCTION OF ENZYMES

FUNGAL AMYLOLYTIC ENZYMES

L. A. Underkofler

Enzymes may be defined as biocatalysts which bring about fic biochemical reactions. In their natural condition, the nes perform metabolic processes of the living cell. Enzymes in every living organism from the highest-developed animals lants to the simplest, unicellular forms of life. Although most e enzymes are endocellular, some are exocellular and are ted outside the living cell. Enzymes are of special importance e fermentation industries, since all fermentation processes are sult of the enzymic activities of microorganisms.

nzymes were used in everyday life and industry to accomplish emical reactions apart from the cell long before the nature and ion of enzymes were understood. The use of barley malt for conversion in brewing and a similar use of crude fungal rations in oriental countries are examples of such ancient use ymes. With the gradual development of knowledge of the e of enzymes, crude preparations from certain animal tissues. s pancreas and the mucosa of the stomach, or from plant tis- such as malt and papaya fruit, were prepared, which readily technical applications in the textile, leather, brewing, and other industries. Once the favorable results of employing en preparations were established, a search began for better, less e sive and more readily available sources of such enzymes. I found that certain microorganisms produced useful enzymes this led to the development of technical processes for making microbial enzymes on a commercial scale.

Takamine, in 1894, was probably the first to realize the te cal possibilities of cultivated enzymes from molds and to intr such enzymes to industry. At present, several concerns are prep and marketing fungal amylases, proteases, and pectinases. Th cussion in this chapter will be restricted to fungal carbohy which act on starch. Other fungal enzymes, as well as bac enzymes, will be considered in Chapter 4.

Many species and strains of molds produce a consid number of enzymes, the amount of the individual enzymes va widely between species and strains. Tauber[illegible] has listed tw three enzymes which have been identified from the mold *Asper oryzae* and twenty from *Aspergillus niger*.

The fungal carbohydrases seem to be exocellular enz These enzymes belong to the class of hydrolases, which bring a hydrolytic reactions. Most of the known carbohydrases are duced by different species of molds, many species forming se different carbohydrases. A remarkable property of the hydr enzymes is their great specificity of substrate; each enzyme re brings about hydrolysis of a particular substrate, but not of ot Among the more important fungal carbohydrases are α-amy limit dextrinase, cellulase, emulsin, inulase, invertase, maltase lactase. Of these enzymes α-amylase, maltase, and probably dextrinase are of industrial importance. α-Amylase is frequ termed the dextrinizing enzyme since it hydrolyzes large s molecules more or less at random with formation of dex Fungal maltase not only converts maltose to glucose, but als drolyzes starch and dextrins producing smaller dextrins and cose. A better designation which is coming into use for this en is amyloglucosidase. Limit dextrinase hydrolyzes malt limit trins with the production of fermentable sugars. A more det discussion of the nature of fungal amylolytic enzymes will be g later.

The absolute and relative amounts of the various enz produced by molds vary markedly between species and even

'n strains of the same species. For example, Le Mense, Corman,
Lanen, and Langlykke[illegible] studied the production of amylases in
nerged culture by three hundred sixty-seven cultures of
ds which included species of the genera *Penicillium*, *Aspergillus*,
zopus, *Mucor*, and *Monilia*. Only eight out of eighty cultures
'enicillium demonstrated α-amylase dextrinizing activity and
was of very low order. Cultures of *Rhizopus*, *Mucor*, and
nilia produced little or no dextrinizing enzyme. Greatest dex-
izing activity was observed in cultures of the genus *Aspergillus*.
wo hundred seventy-eight strains of *Aspergillus* studied, thirty-
produced the dextrinizing enzyme. Highest concentrations of
enzyme were produced by strains of *A. niger*, *A. oryzae*, *A. al-
eus*, *A. foetidus*, and *A. wentii*. There was considerable variation
veen strains of the same species in both α-amylase and maltase
ylogiucosidase) activity, according to Corman and Langlykke.[4]
re recently, Dingle and Solomons[illegible] tested one hundred thirteen
ins of molds by both surface and submerged culture for produc-
of α-amylase as well as other enzymes. These authors likewise
nd great differences in enzyme production between species and
ween strains of the same species.

Not only the difference in strain but also the medium affects
enzyme production markedly, according to Tsuchiya, Corman,
Koepsell,[33] Shu and Blackwood,[25] and Shu.[25a] For the maxi-
n yield of a desired enzyme, a potent mold strain must be se-
ed and the optimum medium for production of the enzyme by
selected strain must be developed.

NATURE OF FUNGAL AMYLOLYTIC ENZYMES

Several of the carbohydrases produced by fungi act on starch
artially degraded starch and thus may be considered the amyloly-
enzymes of these organisms. These include α-amylase, amy
ucosidase (maltase), and limit dextrinase.

According to our present knowledge, α-amylase attacks
,4-glucosidic linkages of large starch molecules, with the forma-
of lower-molecular-weight dextrins. This action results in
d liquefaction and reduction in viscosity of the starch. If the
on is continued long enough, substantial saccharification results,
end product being maltose.

Fungal maltase rapidly converts maltose to glucose, but is also an amylolytic enzyme, since it will hydrolyze starch and de producing lower-molecular-weight dextrins and glucose. Th zyme is now preferably designated amyloglucosidase. Corma Langlykke found that the efficiency of alcoholic fermentat grain was more closely related to the maltase content of the amylase preparation employed as conversion agent than α-amylase content. Subsequently, Corman and Tsuchiya firmed that alcohol yield from grain mashes converted with amylase was positively correlated with the maltase content fungal amylase, but only if an adequate amount of α-amyla also present. Pan, Andreasen, and Kolachov reported that slow secondary phase of alcohol fermentation of malt-sacch grain mashes, the rate of fermentation depended on the r hydrolysis of the dextrins. Addition of fungal amylases g rapid fermentation of the dextrins which was related to the m activity of the enzyme preparation rather than to the conte α-amylase or limit dextrinase. Pool and Underkofler coul no direct correlation between the alcohol yields from grain saccharified with fungal amylase preparations and the al amounts of α-amylase, amyloglucosidase, and limit dextrin either bran or submerged culture preparations of *A. oryzae* strains of *A. niger*.

The enzyme produced by molds which hydrolyzes mal dextrins with the production of fermentable sugars, has been nated limit dextrinase. Very little is known about limit dext It is possible that this enzyme may hydrolyze the α-1,6-glu linkages at the branch points in the amylopectin fraction of s Tsuchiya, Montgomery, and Corman found that over 90 isomaltose could be hydrolyzed by a culture filtrate from *A.* NRRL 330. Isomaltose differs from maltose in that the ch linkage between the glucose residues is of the α-1,6 type in isom whereas it is of the α-1,4 type in maltose. The mold filtrate ously contains an enzyme capable of hydrolyzing 1,6-glucosidi ages. Much additional investigation will be needed to asc whether or not this enzyme is limit dextrinase and to learn the properties and functions of the enzyme.

C

RAW MATERIALS

The raw materials for fungal-amylase production dep rocess employed. Originally, Takamine used steamed rice, h is common practice in oriental countries. He next turned to cereals[27] and then to wheat bran.[28,29] The bran method d superior to other known procedures of producing mold nes and consequently has found extensive application for the uction of commercial fungal enzymes. When bran is used, it oistened with water or dilute acid,[29-30] sterilized, inoculated mold spores, and cultivated in relatively thin layers. For the process, the raw materials are wheat bran, water, hydrochloric and perhaps traces of salts. After growth of the mold on the rate, the enzymes are obtained in aqueous solution by percola systems, followed by precipitation of the enzymes as solid entrates by means of alcohol.

With the development of submerged-culture methods for the luction of antibiotics by molds, similar cultural procedures were essfully developed for making enzymes. In general, different ns of molds may be required for best enzyme production by nerged culture as compared with the bran-culture method. At one concern has produced enzyme concentrates on a large by the submerged culturing of molds. Although undoubtedly largest proportion of commercial fungal enzymes still comes the bran process, it is probable that the inherent advantages e submerged-culture process will in time lead to its eventual cement of the older method. However, Lockwood[15a] has re- ed that efforts of industrial concerns to obtain by submerged ring good, dry, powdered fungal amylase preparations having ame balance of enzymes as from bran cultures have generally unfruitful.

For the submerged process several types of media have been sted and used. The media chosen depend on the uses for h the fungal enzymes are produced. Where fungal amylase is uced in large volumes for saccharification of alcoholic-fermen- n mashes, by-products of the alcohol plant are the favored raw rials. In these cases, the principal raw materials are thin stil- or distillers' dried solubles, and a little ground grain. For the uction of commercial enzyme concentrates, undoubtedly purer naterials are preferred to facilitate the precipitation and re- y of the enzymes. One plant has employed media containing and inorganic salts. Recovery of solid enzyme concentrates then required simply filtration and precipitation of the enzymes by alcohol.

CULTURES AND THEIR MAINTENANCE

Many strains of various species of molds produce carbohydrases. As previously mentioned, the kinds and amounts of the carbohydrases produced depend on the species and strain of mold. Strains of the *Rhizopus* species and of the *Aspergillus* species are the principal ones which have been employed on industrial scale for enzyme production, the second being by far more important. *Rhizopus* cultures have been used in the amylo process for alcoholic fermentation of starchy substrates and *Aspergillus* cultures for production of mold bran and of enzyme concentrates. Of the *Aspergilli,* strains of *Aspergillus oryzae* have been employed most commonly. It is probable that strains of this organism are generally employed by all manufacturers of fungal-enzyme concentrates, using the bran system. It is known, however, that certain manufacturers use other species for the production of special-purpose enzyme concentrates. Strains of *A. oryzae* have been employed for commercial production of mold bran.

It may be expected that as the submerged culture system for fungal-enzyme production expands, selection of strains and species will be made for the production of enzyme concentrates best adapted to specific purposes. Le Mense, Corman, Van Lanen, and Langlykke[14] investigated amylase production by three hundred sixty-seven strains of molds of five genera. Species of *Aspergillus* showed highest amylolytic activity. Of these, several strains of *A. niger* showed higher α-amylase activity when cultivated in submerged culture than the strains of *A. oryzae* tested. Moreover, there was a considerable difference in the activity of the various strains of *A. niger.* Some had much higher α-amylase activity than others, whereas other strains had higher maltase activity. Dingle and Solomons[5a] also found that strains of *A. niger* gave highest yields of α-amylase in submerged culture while strains of *A. oryzae* gave highest yields in surface culture. Corman and Langlykke[4] verified the findings of Le Mense *et al.* and also found that certain species of *Rhizopus,* while almost devoid of α-amylase activity, showed fairly good maltase activity. Therefore, it is quite possible that in the future, enzyme producers will select the mold strains and media employed to produce enzyme concentrates especially suited for diverse uses.

Stock cultures of molds are usually maintained in the laboratory on agar slants of a suitable medium. Various media may be employed. For the growth and isolation of molds, a modification of Czapek's medium[26] has been widely used. Malt-extract medium, yeast-extract sugar medium, and various natural media have had application in cultivating molds. Ideally, for laboratory stock cultures, media should be employed which give good growth and heavy sporulation of the mold cultures. The author has kept cultures of *Aspergillus oryzae* and several other molds for many years by transferring every 2 months on slants containing 2.5% glucose, 2.5% glycerol, 0.5% Difco yeast extract and 1.5% Bacto agar, with storage in the refrigerator after incubating for about 5 days at 30°C to obtain heavy sporulation. Strains of *A. oryzae*, acquired in 1939 and carried on this medium, still have their original growth characteristics and capacity for amylase production. If desired stock cultures may be preserved by means of soil cultures or by lyophilization. These procedures are discussed in Chapter 12.

For plant use, laboratory spore cultures are generally grown on bran medium. A typical procedure is to mix wheat bran with water or dilute hydrochloric acid, distribute in small quantities in wide-mouth flasks or Mason jars, sterilize under steam pressure, inoculate with spores of the mold, and incubate with the flasks or jars laid on their sides. Moisture content of the medium is adjusted as experience dictates, so that the bran dries at the proper rate during incubation. If the bran dries too rapidly, poor spore production results; if the bran does not dry rapidly enough after sporulation is complete, autolysis occurs with a rapid loss in spore viability. One commercial plant[39] produced spore cultures on a medium prepared by mixing 10 g ground corn, 100 g wheat bran, and 60 ml of 0.2 *N* hydrochloric acid, containing 0.62 ppm $ZnSO_4\cdot7H_2O$, 0.63 ppm $FeSO_4\cdot7H_2O$ and 0.08 ppm $CuSO_4\cdot5H_2O$, distributing the moist mass in 10 g quantities in 250-ml, wide-mouth Erlenmeyer flasks, or in 40 g quantities in 2-qt Mason jars, and sterilizing under steam pressure. The cooled, sterile mixture was then inoculated with the mold spores, the bran distributed on one side of the container by gentle tapping, and the flask or jar incubated at 30°C while lying on the side. With this procedure the mold culture grows, sporulates and then dries out. The dry bran spore cultures may be left in the incubator undisturbed for several weeks until it for use.

The laboratory spore stocks are used as starter cultu larger-scale inoculum production in the plant. For the bran p spore inoculation is preferred and large spore stocks to s plant inoculum may be grown on thin layers of bran medi large bottles or jars, or in trays.

Jeffreys[18] described a method of growing plant spore c in shallow metal trays, equipped with perforated covers sterilized and cooled bran is inoculated from laboratory spore the inoculated bran spread in a thin layer in the tray, a p newspaper placed over the top of the tray, and the cover p The newspaper, according to Jeffreys, prevents contaminati absorbs moisture. The trays are incubated on racks in spe cubator rooms, at 78°F, with forced air circulation. After a s incubation period, the tray covers are removed and the mol culture is dried.

Underkofler, Severson, Goering, and Christensen[39] d a similar procedure for tray growing of plant spore culture has been used on a commercial scale. These authors em metal pans 24 × 36 × 4 in. in size. The moistened, sterilize inoculated bran was spread in 1/2 in. thick layers in the steri and the covers put on the pans. The covers were provide air spreaders in the center and air outlets in the corners covers, the pan being placed in a special incubator cabinet. H fied sterile air was passed into the pans through the air sprea the rate of 1,200 to 1,800 ml per minute. When heavy spor had occurred, the covers were removed and the spore culture in the incubation cabinet.

For submerged culturing of molds, mycelial inoculum commonly employed. A laboratory spore culture is used to in flasks of liquid medium which are then incubated on a shak laboratory incubator. The flask shake cultures are emplo inoculate larger flasks or bottles of liquid medium provide aerators. The submerged cultures are incubated with aerati the bottle mycelial cultures employed to inoculate plant seed

Use of vegetative inoculum is a time saver since th culture starts with virtually no lag. As growth starts fro viable particle, the larger the number of viable particles inoculum the faster the development. Maximum efficiency

ed by using a Waring blender for particle-size reduction. The
ne of this procedure was in the gluconic acid process, as re-
d by Moyer, Wells, Stubbs, Herrick, and May.[16] Dorrell and
recently applied it to other organisms. Savage and Vander
k[22] reported that "blended" *Penicillium* submerged mycelium,
ed as much as forty thousand times, adequately substituted for
ended submerged inoculum used at a 10% inoculation ratio.
of the most common faults in submerged mold cultivation is
se of too little inoculum. Not only is the development of the
re delayed, but the desired homogeneous, dispersed type of
th, consisting of small individual colonies or clumps of mycelia,
to develop. Instead, the relatively few viable cells from the
ulum develop into large, individual, usually spherical pellets
h may grow as large as marbles or larger. In most cases, an
ulation ratio of 5 to 10% (v/v) of a heavy submerged vegeta-
culture is most desirable for the plant-scale submerged-culture
ess. The intermediate cultures must, therefore, be successively
ed up in aerated fermentors of increasing size to sufficient
me for the final fermentation.

LABORATORY PRODUCTION

Fungal amylase may be produced in the laboratory by growth of
mold on fibrous substrates, usually wheat bran, or by growth
e mold in submerged liquid culture. Prior to World War II,
irst method was almost universally employed and the enzymic
uct has come to be known as *mold bran*. Since the establish-
t of submerged-culture fungal processes, this submerged tech-
e has been widely adopted for the laboratory cultivation of
ls for enzyme production.

Pioneering investigations on methods for mold-bran produc-
were reported from Iowa State College. Underkofler, Fulmer,
Schoene[28] employed a rotating drum technique and later Hao,
er, and Underkofler[29] adopted a covered aluminum pan with
rated bottom through which air could be passed by alternately
ing air pressure and suction as the mold grew on the moistened
in the container. The laboratory and pilot plant work which
a process for the commercial production of mold bran on the
ge basis has been reviewed by Underkofler, Severson, Goering
Christensen.[30]

The cultivation of molds by submerged culture received pioneering attention since World War II by workers at the Northern Regional Research Laboratory of the U. S. Department of Agriculture, although the procedure was first proposed by Woolner and Lassloffy[42] as early as 1909. For laboratory cultivation on a small scale, the shake-flask technique is employed and on a larger laboratory scale, the mold is grown in submerged culture in flasks or bottles up to 5 gal capacity with vigorous aeration supplied by means of a tube, connected to a suitable air dispenser, which leads to the bottom of the vessel.

Various media have been used for the submerged cultivation of molds for amylase production. Goering, Le Mense, and Ogden successfully employed a synthetic medium containing starch and inorganic salts for both laboratory and pilot-plant work. In a patent, Corman[3a] claimed the use of synthetic media containing starch, a potassium phosphate buffer, compounds such as nitrites and nitrates, aspartic acid, urea, and asparagine as nitrogen sources, and small amounts of salts. In most of the reported work, media containing thin stillage from alcohol fermentation, or distillers' dried solubles, and corn meal have been used. Earlier workers[8,14] found that the addition of 0.5% calcium carbonate to such media was requisite for obtaining maximum yields of amylase.

Tsuchiya, Corman, and Koepsell[34] found that the yields of α-amylase and of maltase depended on the terminal pH of the submerged cultures. This terminal pH should not be allowed to drop below pH 4.2. In the absence of calcium carbonate, the terminal pH of the cultures of *A. niger* NRRL 337 can be controlled by adjustment of the concentrations of the ingredients of the medium, distillers' thin-stillage solids and corn meal. Increasing the thin stillage solids content of the medium results in the rise of the terminal pH with a consequent increase in the yield of α-amylase. Increasing the corn-meal content of the medium results in a lowering of the terminal pH and an increase in the yield of maltase. By adjusting the concentrations of distillers' thin stillage and corn meal in the medium, it is possible to control, to some degree, the yields of both α-amylase and maltase. The concentration variation of the ingredients of the medium affected the production of α-amylase and maltase in cultures of *A. niger* NRRL 330 and *A. oryzae* NRRL 458 less markedly than with *A. niger* NRRL 337.

Tsuchiya, Corman, and Koepsell[34] also found that incorporation of calcium carbonate in the medium, in concentrations previously recommended, results in lowering the yield of maltase. Since the terminal pH can be controlled in the absence of calcium carbonate by using the proper amounts of stillage solids, it was recommended to eliminate calcium carbonate from the medium used for making fungal amylase. A medium containing 5% thin-stillage solids and 5% corn meal gives excellent yields of both ∝-amylase and maltase with *A. niger* NRRL 337.

Recently, Shu and Blackwood[25] have reported a rather extensive investigation on the effect of the variation of carbon and nitrogen sources on the production of fungal amylolytic enzymes by submerged culture. These investigators employed *A. niger* PRL 558 in shake-flask culture. The basal medium contained 0.5% $CaCO_3$, 0.1% KH_2PO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ to which various carbon and nitrogen sources were added. In media containing 1.5% hydrolyzed isoelectric casein several carbon compounds, including polyalcohols, acids and carbohydrates, were tested. The enzyme activities of the culture filtrates varied widely, depending on the carbon source present. Maltose, dextrin, and starch yielded much more ∝-amylase than other compounds used. Maltase and limit dextrinase production were best with these substrates. However, rather good yields of maltase were obtained with glucose and the production of limit dextrinase was least dependent on the substrate.

With starch as a substrate, four types of nitrogen sources (nitrates, amides, ammonium and amino compounds) were investigated. Casein hydrolyzate gave best enzyme yields. Amides did not support good growth. Single amino acids and inorganic nitrogen compounds gave poor yields of enzymes. However, inorganic nitrogen compounds, both ammonium compounds and nitrates, when added to media containing casein hydrolyzate, significantly stimulated the production of ∝-amylase and maltase. This effect was much less prominent when the level of casein hydrolyzate in the medium was raised to 6.0%. Whole proteins gave much poorer results than hydrolyzed proteins. Yields of the enzymes were comparable with enzyme-hydrolyzed casein, gelatin, gluten, and edestin. It was concluded that the availability of the nitrogen source to the organism is one of the most important factors to be considered in the production of fungal amylolytic enzymes.

The relative amounts of ∝-amylase and maltase were controlled by Shu and Blackwood[25] by varying the fermentation time as well as the carbohydrate and protein contents of the media. was found that an increased amount of either carbohydrate or pr tein increases the time required to attain the maximum amylas yield and also increases the maximum yields of all three of th amylolytic enzymes. A culture filtrate of low α-amylase, but hig maltase and high limit dextrinase content was obtained by cultiva ing the mold on a medium containing 2% starch and 6% casei hydrolyzate for 6 to 8 days. A low maltase preparation, with fa α-amylase activity, was obtained by culturing for 48 hours on medium containing 3% starch and 4% casein hydrolyzate. Highe yields of all three amylolytic enzymes were obtained by culturin for 6 days on a medium containing 8% starch and 4% casei hydrolyzate.

Shu[24a] more recently reported further studies on nitroge sources for the production of amylolytic enzymes by submerge culture of *A. niger.* He found that amino acids or proteinaceou substances are not required for production of high yields of th enzymes. A readily available nitrogen source, preferably a potenti alkali donor, such as ammonium acetate, should be used for hig yields of α-amylase. Accumulation of maltase and limit dextrinas was suppressed by maintaining a high pH (about 8) durin fermentation.

Another noteworthy contribution made by Shu[24b] has bee the design of a closed shake-flask fermentor which simulates co ditions in shake-flask culture in a cotton-plugged Erlenmeyer flas In this apparatus, the uptake of oxygen during a fermentation recorded automatically. In the past, oxygen utilization could n be followed in shake-flask cultures. This was a distinct disa vantage since information on substrate utilization, including th of oxygen, and product formation under various conditions necessary if optimal fermentation conditions are to be establishe Shu's equipment and procedures should be very useful to invest gators developing new aerobic submerged-culture fermentations all types.

Shu investigated the oxygen requirements of three different su merged fermentations with his apparatus, including fermentatio producing citric acid, ustilagic acid, and α-amylase. For th α-amylase fermentation he employed *A. niger* PRL 558 and medium containing 3% soluble starch, 2% yeast extract and 0.5 calcium carbonate. The mycelial growth was filamentous an mushy. The oxygen uptake curves showed that, after the initial lag phase, the rate of oxygen uptake decreased with time. This was more pronounced at reduced oxygen tension. The maximum efficiency of enzyme synthesis by the mycelium occurred at an oxygen uptake rate in the neighborhood of 20 millimols per hour per liter.

THE PLANT FERMENTATION PROPER

Bran Process

Little has been published on the exact procedures employed by the plants producing industrial fungal enzymes. Takamine was the first to use the bran process and secured some nineteen patents on various aspects of the production and use of fungal enzymes. He particularly recommended[32] and patented[31] the use of a rotating horizontal drum. In the drum method, the bran is moistened with an approximately equal weight of water and sterilized in the drum by steaming while the drum is rotated. The bran is then cooled by means of a stream of cool air, inoculated by blowing in dry spores of the mold *Aspergillus oryzae,* and incubated. During the incubation period, a stream of cool, humidified air is passed through the drum as it rotates. The movement and air stream serve to dissipate the large amount of heat formed by oxidation of carbohydrates by the fungus. Water may also be sprayed on the outside of the drum to prevent the temperature from rising to a level inhibitory to the growth of the mold.

It is known that some industrial enzyme plants currently employ drums, but other manufacturers who have used drums have abandoned them in favor of tray or cabinet culturing methods.[15a] In the drum process, it is difficult to control the temperature effectively, there is danger of contamination, and the rubbing action of the bran particles, as they are tumbled in the rotating drum, damages the delicate mold mycelium during the early stages of growth. However, with careful control, excellent enzyme production is achieved in the plants employing the drum system.

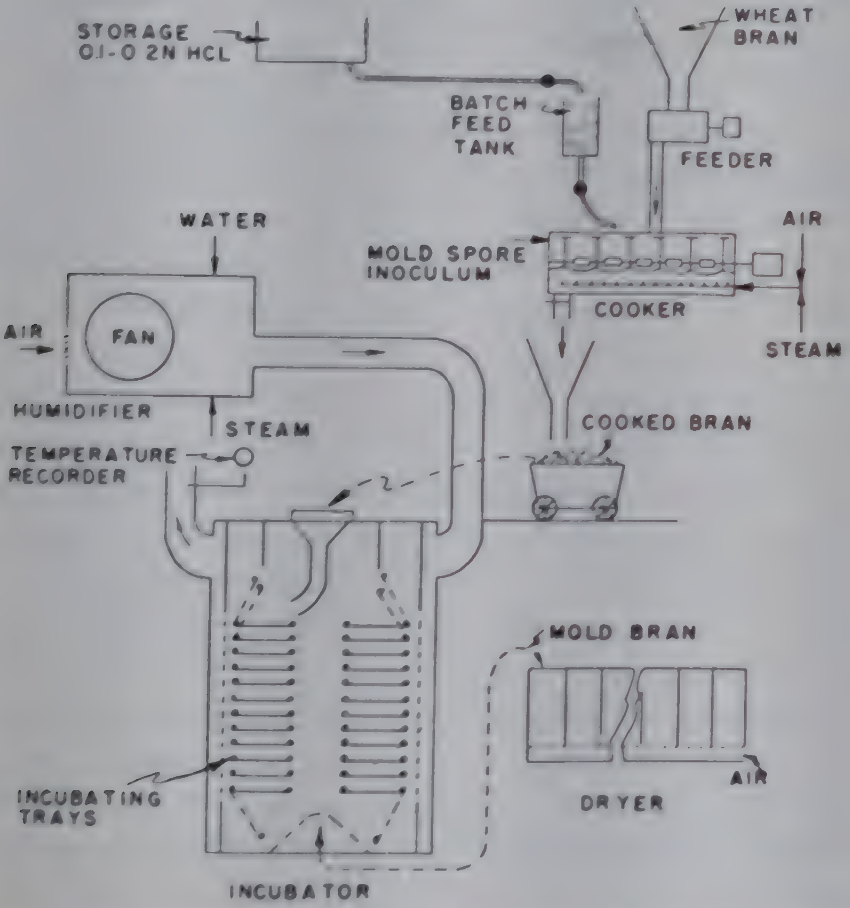
Shellenberger[24] has briefly described a typical commercial method for preparing fungal enzymes on wheat bran. The moistened bran is first cooked for about 1 hour in a rotating cooker by direct steam under pressure to gelatinize the starch and sterilize the mash. The cooked bran is then cooled to about 40°C in a waterjacketed cooler and prepared for transfer to the growing chamb by adjusting the pH (lactic acid may be used), adding miner salts if necessary, and inoculation with mold-spore culture.

The inoculated bran is then spread out in layers in the gro ing compartments and optimum growth conditions maintained regulating the temperature. Temperature control is the m important of the process variables, but the prevention of contamin tion in the incubation chambers is also of major importance. It usually necessary to warm the molding bran layer at the beginni and to cool when growth becomes rapid. Temperatures may maintained at 32°C for the first 24 hours, but when the cake begi to heat as a result of active growth, the temperature of the growi compartment is reduced to aboue 26°C. After 4 days, the mo mycelium has matted the medium together and enzyme producti has reached a maximum.

Moist culture, on removal from the growing chambers, is dri in either rotary or tray driers. Highest temperature of the m should not exceed 50°C, because, if subjected to higher temperatu the enzymes would be inactivated. Initial temperature depends moisture content of the culture, type of drier, and size of charg After drying, the mass is broken up by grinding and may marketed as such or processed further for production of enzy concentrates. For this purpose, the mold bran is extracted wi water, filtered, and the enzyme precipitated from the filtrate by su cessive additions of ethanol. The precipitate is then separated fro the liquor by centrifuges, dried in a vacuum drier, being caref not to exceed 55°C, and the dried product ground and blended obtain a final product of uniform activity. Shellenberger[24] stat that in addition to amylase, other enzymes can also be manuf tured by this or similar methods. These include protease, pr topectinase, and pectinase (Chapter 4).

For the bran process, shallow trays undoubtedly have h widest application. Since details of the processes employed by co mercial enzyme concerns are not available, those interested in larg scale production of fungal enzymes, for such uses as saccharifyi agent in alcohol production and as new producers of commerci enzymes, have been compelled to develop their own procedur and some of these have been published. Undoubtedly, the concer manufacturing commercial fungal enzymes employ similar process e variations probably being only in the manner of handling trays, the types of incubation chambers employed, etc.

Perhaps the most extensive investigation of methods for culti- ating the mold, *Aspergillus oryzae*, on bran for large-scale produc- ion of mold bran was reported by Underkofler, Severson, Goering, nd Christensen.[39] This process proved satisfactory for the plant roduction of mold bran at the rate of 10 tons a day. The process low sheet for this plant is shown in Figure 5.


STORAGE
0.1-0.2N HCL
WHEAT
BRAN
BATCH
FEED
TANK
FEEDER
WATER
AIR
MOLD SPORE
INOCULUM
COOKER
STEAM
AIR
FAN
HUMIDIFIER
STEAM
COOKED BRAN
TEMPERATURE
RECORDER
MOLD BRAN
INCUBATING
TRAYS
AIR
DRYER
INCUBATOR

IGURE 5. *Flow Diagram of Mold-Bran Production at Eagle Grove, Iowa*

In this process, the bran was fed batchwise into the cooker nd mixed by means of the cooker agitator with the requisite mount of 0.2 *N* hydrochloric acid, the proportions being in the eighborhood of 2 parts bran to 1 part acid. The moisture content as varied in accordance with experience so that condensation of team during cooking should yield a mash containing approxi- ately 50% moisture. The moist bran was sterilized in the cooker y injecting steam directly into the mash for 30 minutes with con-

tinuous agitation. At the end of the heating period, the ste
shut off and air injected, with the agitators running. This
cooled the mass by evaporation. When the mix had been co
about 35°C, the air was shut off and spore inoculum fed i
cooker by means of a dusting atomizer and air stream. Whe
oughly mixed, the batch of inoculated bran was conveyed
incubation rooms. Here the inoculated bran was spread
layers on trays having hardware cloth (wire screen) bottom
trays were hinged lengthwise from the wall and filled fr
bottom trays to the top until all trays in a room were fille
temperature of the incubating mold bran was controlled
tinuous circulation of humidified air over and under the t
means of air ducts in the walls of the incubation rooms.
conclusion of the incubation period, the trays were dropp
at a time, to dislodge the mold bran cake, beginning with
tom trays. The moist mold bran was conveyed by mec
conveyors to bins with perforated bottoms. Here it wa
by means of a current of warm air, blown up through the m

Jeffreys[illegible] has described a tray method employed in
plant. In this plant, mechanical handling is provided for a
as is possible. The bran is fed by a percentage feeder on
moving conveyor, where it is mixed with the required am
water, heated with steam to at least 190°F, and the steami
tinued for 15 minutes. The steamed mixture is then drop
a second conveyor, where it is cooled by means of a current
filtered air. The prepared substrate is inoculated by spra
suspension of spores in sterile water on it. At the same ti
also inoculated with dry spores fed by means of a percentage
and blower.

The substrate and spores are thoroughly mixed and d
on a spreader which distributes it uniformly in sterilized tra
loaded trays are conveyed to a truck which holds twenty tra
apart. The loaded trucks are pushed into the culturing tu
overhead trolleys. Cool, humidified air is circulated in the cu
tunnels to maintain the temperature of the growing mol
slightly below 37°C. If it is necessary to warm the air at the
ning of the culturing period, this is accomplished by ste
controlled by thermostat.

During passage of a truck through the culturing tun
mold growth reaches an optimum in 24 to 30 hours and the

en transferred to the drying tunnels. Warm dry air is circu- through these tunnels. The air flows parallelly to the direction ich the trucks are moving in the primary stage and counter- nt to their direction in the secondary stage. During drying, oisture content is reduced to about 8% and the temperature product is not allowed to exceed 110°F. The dried mold s ground and bagged. For preparation of enzyme concen- the mold bran may be extracted either before or after drying. fter the contents of the trays are dried, they are removed the truck and dumped. They are then carried inverted on a belt over revolving brushes which clean them. They are next d through an oven at 300°F for 3 minutes. After being auto- ally turned, they go to the spreader for reloading with a fresh of inoculated bran substrate.

Submerged Process

here is no description in the literature of the commercial ction of enzymes by submerged culturing of molds. However, al papers have appeared on the submerged culturing of molds roducing diastatic material for the saccharification of alcohol- ntation mashes. This procedure was first proposed by Wool- nd Lassloffy[12] as early as 1909. Erb and Hildebrandt[7] grew erged cultures of *Rhizopus delemar* in plant-scale quantities se as adjunct with malt in saccharifying alcohol-fermentation es. More recently, a number of investigators[1,6,8,16,18] employed erged cultures, especially of *Aspergillus niger* NRRL 337, to y replace malt for the saccharification of grain-fermentation es. This work furnished the basis for commercial-scale experi- al units for submerged fungal amylase production and success- lant-scale application, in the grain-alcohol distillery of the Processing Corporation, Muscatine, Iowa.[2,3,10]

he author has had close contact with the pilot-plant and ommercial production of fungal amylase concentrates by sub- d culture at one commercial concern. This work was con- d for the development and production of a concentrate high amylase and as low as possible in proteolytic activity. One m employed[8] was a starch, inorganic salts medium which ned, per liter, 30 g cornstarch, 10 g KNO_3, 5 g $CaCO_3$, 0.8 g O_4, 0.4 g $MgSO_4$, 0.4 g KCl, 0.0035 g each of $FeSO_4$, $ZnSO_4$, and $CuSO_4$, and 3 ml oleic acid. The culture employed was a selected strain of *Aspergillus oryzae*. Laboratory spore cultures were carried in the usual manner in flasks on bran medium. From this laboratory culture, spore inoculation was made into flasks of liquid medium which were incubated on a reciprocal shaker at 30°C. After about 72 hours, the shake-flask cultures were employed to inoculate 2 gal of liquid medium in 2.5-gal, tall-form pyrex bottles and incubated at 30°C, with vigorous aeration, sterile air being introduced through a glass tube leading to the bottom of the bottle where it was dispersed in bubbles through a cloth sack of several thicknesses. After incubation for 24 hours, a bottle of culture was used to inoculate 60 gal of medium in a glass-lined seed tank. The contents of the plant seed tank, after 24 hours of incubation with aeration, were used to inoculate the production fermentor. Two types of production fermentors were used. In the glass-lined type, 300 to 600 gal of liquid were employed and in the steel fermentors, 600 to 1,200 gal of liquid.

The seed tanks and production fermentors were equipped with agitators and air spargers in the bottom of the tanks. Air was provided under pressure and was sterilized by passage through carbon filters.

The course of the fermentations was followed by periodic sampling. Samples were inspected microscopically for bacterial contamination and analyzed for α-amylase activity by the method of Sandstedt, Kneen and Blish.[21] When the maximum α-amylase activity had been reached, as shown by the analyses on consecutive samples, the aeration was stopped and the fermentor contents processed to obtain the amylase. Depending on a number of factors, such as medium composition, rate of aeration, and amount of inoculum, the maximum amylase content was reached in 64 to 135 hours in individual fermentations. These were considered successful when α-amylase activity amounted to about 75 SKB units (30°C) per ml. Occasional potencies as high as 140 units per ml were obtained.

The amylase was recovered from the fermented liquors by separating the liquid from mold mycelium by means of filter presses, concentrating the liquid about fourfold by vacuum evaporation, and precipitating the enzyme with 1.6 to 2.3 volumes of isopropyl alcohol per volume of concentrated filtrate at pH 7.5 to 8.5. Under the optimum conditions almost quantitative recovery of the α-amylase was obtained in the precipitates from the alcohol treatment. The solid enzyme concentrate was dried in a vacuum drier, and powdered by grinding. Enzyme concentrates containing more than 6,000 SKB units per g were made. All preparations were standardized at 3,000 SKB units per gram by dilution of the solid enzyme concentrates with inert ingredients. For food products, dilution with starch and/or dextrin was satisfactory. For nonfood uses where an insoluble residue was not objectionable, diatomaceous earth could be the diluent.

INDUSTRIAL APPLICATIONS AND ECONOMICS

No information is available as to the costs of production of commercial fungal amylases. The fungal amylase concentrates marketed are rather expensive in terms of unit weight, but in terms of unit activity are not more costly than competing products obtained from other sources, such as pancreatin, malt, and bacterial preparations.

The largest potential use for fungal amylases is as saccharifying agent for grain-alcohol fermentation mashes. Slightly better alcohol yields result from the use of fungal amylases than from malt for this purpose. Tables 10 and 11 in Chapter 2 of Volume I give a comparison of alcohol yields obtained from grain mashes saccharified by malt, mold bran, and submerged fungal amylase preparations. The use of mold bran on the commercial scale has been shown by Underkofler, Severson, and Goering[38] to effect savings over malt. Le Mense, Sohns, Corman, Blom, Van Lanen, and Langlykke,[15] and recent publications on plant-scale tests[3,40] have estimated a savings of 6 to 10¢ per bushel of grain mashed or 3 to 4¢ per gallon of alcohol produced by utilizing mold-amylase liquor instead of malt.

Fungal amylases, as well as amylases from other sources, have many and varied industrial and practical applications in addition to their present and potential use in the grain-alcohol fermentation industry. Some of the important uses, with literature references, are discussed in more detail in Chapter 4.

The first important market for fungal amylase concentrates was for the pharmaceutical trade, and this continues to be an important outlet.

Fungal amylases are extensively used in the food industries.

Usually highly saccharifying types of fungal amylase concentrate which contain α-amylase and amyloglucosidase, are employed i manufacturing various food products. However, they are also use for removal of starch, which may cause cloudiness, from fruit e tracts, and in the production of pectin from apple-pomace. For th last use, the enzyme preparation must not contain pectolyt enzymes.

One important industrial application of fungal amylases in the conversion of acid-modified starches to sweet sirups. Lang lois[13a] has recently discussed the application of enzymes in cor sirup production. Acid hydrolysis of starch is a random actio and the composition of the resulting sirup is a function only the degree of conversion. Enzyme hydrolysis is a patterned actio and the composition of the resulting sirup is a function both the degree of hydrolysis and the kind of enzyme used. The thre diastatic enzymes, fungal α-amylase and amyloglucosidase, an malt β-amylase, show distinctly different patterns of hydrolysi One or more of these enzymes may be used in conjunction wit acid in the hydrolysis of starch to produce sirups of very specif composition and having definite physical and chemical characte istics. Sirups differing in sweetness, fermentability, viscosity, osmot pressure, and humectant character can be produced as desire Three products have been produced commercially. One is cor verted to about 63 D.E. (dextrose equivalent) and has high swee ening power with no crystallizing tendency. Another is converte to about 55 D.E. and has moderate sweetness and high ferment bility. A third is converted to over 90 D.E. and has a degree sweetness almost equal to pure glucose. It crystallizes to a soli mass and is palatable without further purification. Corn sugars this degree of conversion prepared by acid hydrolysis alone are n palatable because of bitter reversion products.

Recently fungal-amylase concentrates have been suggested an extensively tested for fortification of flour. Johnson and Miller[12, reviewed the status of fungal enzymes in the baking industry. Th reported that new standards of identity for bread, proposed by th Federal Security Agency after extensive favorable hearings, allo the addition of enzyme preparations from *A. oryzae* to bread dough They give the following advantages in using fungal enzymes: r duction of "buckiness" in doughs by increasing their mobility du ing fermentation; control of dough consistency; and improveme loaf volume and of crumb characteristics, including increase in ›ftness of crumb and elimination of possible sticky crumb due to ı excessive amount of supplements. They state that today, fungal ızyme supplements are available with any desired ratio of proeinase to amylase, giving new control over doughs prior to make-up.

More recently Skovholt[26a] has discussed the use of fungal nzymes in bread production, and Reed[19b] the manufacture of inlustrial fungal enzymes for use in bread making. Pence[17a] in reviewıg the current status of problems in the panary fermentation ndicated that recent advances in the knowledge of the action of mylases and proteinases on flour components during fermentation ınd baking have stimulated a rapidly increasing use of fungal nzymes in bread production.

In the textile and paper industries, dextrinizing fungal amylıse concentrates may be employed for converting starches in preparing sizing materials, although greater heat stability gives bacterial ımylase preference for these uses. Such modified starches are used or sizing cotton and other textile fibers before weaving and in paper coatings. For removing starchy sizing from fabrics after weaving, fungal amylases are frequently employed. The properties of the dextrinizing enzyme make it also suitable for the production of dextrin-type adhesives.

Amylases, mixed with proteolytic enzymes, are employed in dry-cleaning preparations. These agents are used for hand spotting small food stains before cleaning. For badly soiled articles, it is customary to immerse the garment in an enzyme bath which removes the starch or gelatin size and with these the stains and to resize the article after cleaning. Although probably none of the commercial products sold for this purpose contain fungal enzymes, limited tests have shown mixtures containing fungal amylase to have merit.

COMPETITIVE OPERATIONS AND PROCESSES

Fungal amylases, being enzymes which solubilize starch and eventually convert it to sugar, are directly competitive with other amylolytic substances. Acid hydrolysis is, of course, probably the cheapest method of converting starch. Therefore, it has long been employed in preparing starch sirups and sugars, for example, corn sirup and glucose. Much fungal amylase is used in the corn-sirup industry to hydrolyze residual dextrins after acid hydrolysis a thus increase the maltose and glucose content of the sirups.

In the fermentation industry, malt enzymes have been u conventionally in this country for saccharifying starchy fermen tion mashes, such as those from grains and potatoes. Fungal enzy have been shown to be at least equivalent and probably super to malt for this purpose. Mold bran has been employed in an dustrial-alcohol plant and submerged fungal enzyme has be studied on a commercial scale in at least one plant for this purp as mentioned previously. (See also Chapter 2 of Volume I.)

Malt amylases are also competitive with fungal enzymes desizing textiles and for preparing sizes for fibers and paper. H ever, for the latter purpose, malt is at severe disadvantage as c pared with fungal enzymes since malt enzymes produce more ra initial saccharification rather than dextrinization.

Bacterial amylases are also directly competitive with fun amylases in several applications. Where only dextrinization required, bacterial amylase is usually preferred to fungal amyl This is particularly true where relatively high temperatures an pH near the neutral point are necessary. For use at lower tempe tures and in the more acid pH range, fungal amylase is superi Several of the industrial concerns which produce fungal enzy also produce bacterial enzymes. From these sources, a range microbial amylase products is available which fit a wide variety processing conditions and requirements. From this large gro of fungal and bacterial enzymes of different properties, the vari can be selected which is most suitable for any particular purpose

MINOR CONSIDERATIONS

The availability of fungal carbohydrases has contributed mu to our understanding of starch structure and of the mechanisms the degradation of starch by enzymes. Progress in the knowledge the amylolytic content of fungal-enzyme preparations has been ra in recent years. At one time, it was considered that the princi amylolytic enzyme of fungi was α-amylase and it was well kno that little or no β-amylase, so characteristic of cereal grains a malt, was present in fungal amylase preparations. Recently the i portance of fungal amyloglucosidase in starch splitting has be emphasized. Schwimmer,[23] Corman, and Langlykke[4] and Corm

d Tsuchiya[5] have demonstrated the importance of this factor
the production of fermentable sugars from starch by fungal-
zyme preparations. The fungal amyloglucosidase, frequently
esignated as "maltase" or "glucogenic enzyme,"[4] is not specific for
altose, but also produces hydrolysis of starch and glucose oligo-
ccharides having 1,4-glucosidic linkages. Recently Phillips and
aldwell[18] have attempted the purification of this enzyme—which
ey have called "gluc amylase"—from *Rhizopus delemar* and ob-
ined an enzyme solution virtually free from α-amylase. They[19]
udied the action of the enzyme on a number of substrates. The
luc amylase produced 100% of the theoretical glucose from maltose,
5 to 96% from the linear and branched fractions of corn starch and
f waxy maize starch, 92% from glycogen, and 89% from residual
eta dextrins. The enzyme did not attack alpha or beta Schardinger
extrins, isomaltose (brachiose), or a bacterial dextran having
redominantly 1,6-α-D-glucosidic linkages.

Another carbohydrase produced by fungi is limit dextrinase,
hich hydrolyzes limit dextrins resulting from extended action of
nalt or bacterial amylases on starch. It has been postulated that
he main function of this enzyme is the hydrolysis of 1,6-glucosidic
inkages. However, there is no experimental evidence that this is
he case.

Investigators are attempting the preparation and purification
f the fungal carbohydrases and when these will become available
n pure form, they should make possible even more rapid strides
n the understanding of enzyme function and starch structure.
chwimmer,[23] Roy, and Underkofler,[20] and Phillips and Caldwell[18]
ave described the purification and concentration of fungal amylo-
lucosidase. Recently Underkofler and Roy[37] reported the isolation
nd crystallization of α-amylase and of limit dextrinase from a
ubmerged-culture liquor of *Aspergillus oryzae*. A little later
ischer and de Montmollin[8a] announced the purification and crystal-
ization of α-amylase of *A. oryzae* from commercial takadiastase.

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CHAPTER 4

MICROBIAL ENZYMES OTH THAN FUNGAL AMYLASES

J. C. Hoogerheide

Enzymes may be defined as biocatalysts which bring abou their natural habitat, specific biochemical reactions forming of the metabolic processes of the living cell. Enzymes are h specific in their action on substrates and often many diff enzymes are required to bring about, by concerted action, sequence of metabolic reactions performed by the living cell

Certain enzymes may be isolated from the living cell an tain their specific activities *in vitro;* others, however, have no been separated. Several enzymes have been purified and obta in crystalline form; they were found to be protein in nature composition of which did not differ significantly from tha nonenzymic protein. It is not known at present what mak protein an enzyme, but it is significant that certain investig believe that practically all protein present in microorganisms, as yeast, is enzyme protein.[42]

Only a relatively few enzymes have been studied intensi Best known are the hydrolases, which bring about various drolytic reactions. The enzyme systems which catalyze the synt occurring in living cells are still largely unknown, although in gations are now quite active in this field.

The practical application and industrial use of enzymes to
mplish certain biochemical reactions apart from the cell dates
k many centuries and was practiced long before the nature or
tion of enzymes was understood. The use of malted barley for
ch conversion in brewing, the addition of saliva to starchy prod-
by primitive tribes in preparing fermented liquors, the use of
g for bating or puering of hides in leather making are examples
ncient uses of enzymes. It was not until the turn of this century
t the agents became known which are responsible for bring-
about such biochemical reactions. Crude preparations from cer-
animal tissues (pancreas, mucosa of the stomach) or from
nt tissues (papaya fruit) were prepared which found technical
lications in the textile, leather, and brewing industries. Once
favorable results with such enzyme preparations were estab-
ed, a search began for better, less expensive, and more readily
ilable sources of the enzymes. It was found that certain micro-
anisms excrete enzymes similar to the amylases of malt and
creas, or to the proteases of the pancreas and papaya fruit.
is led to the development of processes for the production of such
robial enzymes on a commercial scale.

Takamine,[88] in 1894, was probably the first to realize the techni-
possibilities of cultivated enzymes and to introduce them to
lustry. Whereas this investigator was mainly concerned with
ymes from fungi, Boidin and Effront,[14,15] in 1917, pioneered in
production of commercial enzyme preparations from bacterial
rces.

Technological progress in this field during the last decades has
n so great that for many uses, cultivated enzymes have replaced
animal or plant enzymes, such as pancreatin, papain, or ficin, in
lustrial applications. At present only a relatively small number
microbial enzymes have found commercial application, chiefly
amylases and proteases of bacteria and molds, the invertase from
st, and the pectinases and tannases from mold species. With
reasing knowledge of enzymes, this field undoubtedly will be
ch expanded in the near future.

MICROBIAL AMYLASES

Classification of Amylases

Amylases are conventionally divided into α- and β amylases.

∝-Amylases are capable of breaking down the structure the starch molecule at random into large fragments (dextrin consisting of a series of glucose anhydride units. This type of brea down causes a tremendous drop in the viscosity of a starch soluti and leads to a rapid disappearance of the typical blue colorati of starch on addition of iodine. Only when this type of attack h been accomplished are the dextrins further broken down in smaller fragments by this enzyme. The rate of attack on dextri however, is much slower than that on starch. ∝-Amylase is p marily a starch-liquefying and starch-dextrinizing enzyme.

β-Amylases attack the starch molecule from the ends onl cutting off maltose units until their action is blocked by a si chain or phosphate group in the starch molecule. Then the enzym cannot attack the residue further, except when a new end is create for example, by the action of an ∝-amylase. β-Amylase is primari a saccharifying enzyme; it is found exclusively in plants bei especially abundant in some ungerminated grains, malts, and swe potatoes.

Both bacterial and mold amylases are ∝-amylases, though no means identical. Microbial amylases are widely different for ea species, or even strain, especially in their attack on dextrins and the ultimate amount of fermentable sugars formed from starch.

The industrial significance and the manufacture of mo amylases have been discussed in the preceding chapter and, ther fore, only the amylases of bacterial origin will be discussed here.

Bacterial Amylases

BACTERIA WHICH PRODUCE AMYLASE

When certain strains of bacteria are streaked on a nutrie agar medium containing starch, often a colorless halo may be o served around the colonies when dilute iodine is poured over t plate after completion of the growth process. From the many speci and strains which show this phenomenon, relatively few have bee investigated as to the type of enzyme they excrete. The followi species have been studied in recent years: *Bacillus subtilis* a related species, *Bacillus diastaticus*, *Aerobacillus macerans*,[11,12,] [87,88,89] *Aerobacillus polymyxa*,[79,88] *Clostridium acetobutylicum*,[37] and other butyric acid bacteria, *Bacterium cassavanum*,[85] *Ph tomonas destructans*,[17] and *Actinomyces microflavus*.[17]

From these species, only *B. subtilis* is known to be used in this country, at present, for the commercial production of bacterial amylase. However, in Russia, *B. diastaticus* seems to have found commercial application for amylase production.[39]

AMYLASE PRODUCTION BY *Bacillus subtilis* IN TRAY FERMENTORS

Source and Selection of Cultures. *B. subtilis* is one of the most widely distributed bacterial species; it may be isolated with little difficulty from soil, dust, air, surface water, or compost from almost any part of the world. An interesting study of a large number of strains of this organism, isolated from different sources, was made by Peltier and Beckord[70] and by Kneen and Beckord.[48] These investigators found that strains of *B. subtilis* can be divided into two distinctly different groups. The great majority of strains isolated from soil or from plant material varied greatly in amylase production from none to relatively high levels. The amylases excreted by these strains were of the saccharifying type and gave rather pronounced conversion of starch to fermentable sugars. However, the yield of the amylase excreted by these *B. subtilis* strains is low and such enzymes have not found industrial application as yet.

However, practically all strains of *B. subtilis* isolated from ropy bread were able to excrete large amounts of amylases. These amylases were of the nonsaccharifying type, with a very high starch-liquefying and dextrinizing activity, but forming little fermentable sugars even after 24 hours of incubation. It is this type of bacterial amylase that is produced commercially and has found wide industrial application. Besides ropy bread, suitable strains for the production of this amylase may be isolated from grains, flours, and other starchy products.

The selection of a suitable strain is of the utmost importance for successful amylase production. Such factors as yield, time required to obtain maximum yield, composition of the medium, reproducibility of results, ease of filtration and purification, properties and stability of the final product are all important in determining the choice of the strain. Once this selection has been made, all possible care must be taken to prevent degeneration, since it is a well known fact that strains of *B. subtilis* are subject to considerable variation beyond the control of the investigator. Lyophilized cultures as well as spore cultures in dry sterile soil (see

Chapter 12) are often used for maintenance and preservation of
stock culture in its original condition.

Medium for Commercial Production of Amylase. It i
rather generally accepted rule in enzymology that production
specific enzymes by microorganisms is stimulated when the cult
medium in which the organism grows contains the substrate to be
tacked by the enzyme. It is assumed that enzymes are produced
microorganisms for the modification of potential nutrient substr
to bring them into a form which can be assimilated by the organi
Frequently an enzyme may not be produced in any considera
amount by an organism unless the presence of a potential nutr
source requires it. However, amylase production by *B. subtilis* c
not be explained by such a hypothesis. It has been pointed out
Boidin and Effront[14,15] that abundant amylase secretion occurs in
complete absence of starch. In fact, many excellent amylase-p
ducing strains are not able to utilize the breakdown products
their amylases and no real reason can be advanced why such stra
excrete an abundance of amylase.

Whereas addition of carbohydrates, including starch, to
medium is not essential for the formation of amylase by *B. subt*
a prerequisite for good amylase production is a high percentage
assimilable protein, preferably of plant origin. Defatted soyb
flour, peanut or linseed press cake, casein, distillers' slop, aspara
butt juice, dried brewers' yeast, corn-steep liquor, or a mixt
of such ingredients, in 5 to 10% concentration, were found to g
excellent results and are used most frequently for commercial p
duction of *B. subtilis* amylase.[1,6,14,15,80,81,97,98] In order to make m
of the insoluble proteins of the previously mentioned substr
available to the organism, the suspension is first hydrolyzed w
boiling dilute acid or by enzymic treatment. Often after neutral
tion, about 0.1% each of dipotassium phosphate, calcium chlor
and magnesium chloride is added as well as traces of manganese a
iron salts. The medium is then filtered, the pH adjusted at 6.5
7.0, and sterilized for 30 to 60 minutes at 15 lb steam pressure.

The equipment used for sterilization of the medium is p
erably glass-lined or stainless-steel pressure vessel provided w
agitator and with a jacket or coils for heating and cooling. Batc
up to 1,000 gal are prepared for commercial amylase producti
depending on the size of the culture vessels. Extreme care m

taken that no contaminating organisms can enter the tank dur- cooling and a slight positive pressure is maintained in it at times.

After cooling to 35°C, the medium is inoculated with a pure ture of *B. subtilis.* The inoculum may be prepared in bottles ntaining the same nutrient medium as mentioned previously d is grown for 1 to 3 days at 30°C to 35°C. About 1 l of inoculum sufficient for 1,000 gal of medium. The inoculated culture is then dy for transfer to the special culture vessels.

The Importance of Pellicle Formation during Growth. *subtilis* grows on the surface of a nutrient medium forming a re or less heavy, wrinkled pellicle; very little growth occurs in medium itself underneath this pellicle. On incubation at 30° 35°C, the pellicle becomes visible often within 24 hours. It in- eases in thickness and reaches its maximum within 2 to 3 days. n longer incubation, the gray-white, wrinkled pellicle starts to tolyze, becomes very brittle, and abundant spore formation can observed.

Boidin and Effront,[14,15] in 1917, stressed the importance of a od heavy pellicle for amylase production. When the pellicle is sturbed and becomes submerged, little amylase is formed unless a w pellicle is produced. Gentle circulation of the medium under- ath the pellicle promotes amylase excretion, but is difficult to complish on a commercial scale without danger of damage to the llicle.

The thickness of the nutrient layer is of critical importance, 5 to 4 cm being most commonly used. A thinner layer usually ves somewhat higher yields, but reduces the over-all yield of a rmentor. A bacterial pellicle does not continue to produce nylase, even when the nutrient medium underneath is replaced a fresh medium.[8] Usually production ceases within 6 days. This one of the main reasons why the depth of the nutrient layer nnot be increased above a certain level without merely diluting e total amount of amylase produced.

Culture Vessels. In order to obtain the large surfaces re- ired for good surface growth of *B. subtilis*, special culture vessels d to be designed. The equipment proposed by Boidin and front[14,15] was found to be very suitable and is still used, although nsiderably improved in detail.[1,97,98] It consists of a vertical,

circular tank with removable head. The dimensions of the cultur
tanks range up to 8 ft in height and 6 ft in diameter. A heav
shaft goes through the center and passes through a stuffing box i
the tank head. Fastened to this central shaft is a series of shallo
circular trays, spaced one above the other and filling the tank fro
top to bottom. Each tray has an overflow enabling the operato
to fill all trays from the sterilizer, starting with the top tray an
gradually descending until the bottom tray starts to spill over.

The culture vessel, together with the connecting line to th
sterilizer and an adjacent air filter, can be sterilized with live stea
under pressure before the charging operation. The air filter pr
vides sterile air during cooling and later during the growth proces
When cool, the culture vessel may be charged with the inoculate
medium by applying a slight overpressure with sterile air in th
sterilizer.

Propagation and Harvesting. Since *B. subtilis* is an obliga
aerobic organism with a high growth rate, a large amount
oxygen is consumed and carbon dioxide is produced during th
growth period. In order to supply this oxygen demand and t
eliminate dissimilation products, it is necessary to circulate fres
sterile air across the pellicle during active growth. This can b
done by means of a pipe system with outlets over each tray. A sligh
overpressure is maintained in the culture vessel throughout the ru
to reduce the danger of contamination. The temperature is mai
tained at 25° to 30°C. During the active growth period, muc
heat is developed which must be dissipated either by cooling th
circulating air or by equipping the tank with a suitable jack
for water cooling. Incubation is continued for 3 to 7 days,
which time, maximum potency is reached. The pH gradually i
creases from 6.5 to about 8.5. It is not advisable to continue th
incubation too long, since a slow but steady decrease in poten
occurs at this relatively high pH.

Harvesting is done by rotating the shaft, to which the tra
are attached, by means of a crank on the tank head, thus spillin
the liquid over the edges of the trays and collecting it via th
bottom valve of the tank. In order to remove bacterial debris, th
culture is then filtered by means of a plate-and-frame filter-pre
or by centrifugation.

The Final Product. The clear liquid obtained from filtratio of the fermented beer is concentrated from three to ten times, depending on the potency of the culture liquid and the final potency desired. Heating above 50°C for prolonged periods should be avoided during this concentration process; the pH should be maintained close to neutrality.

Usually the enzyme is marketed as a liquid concentrate. In order to prevent putrefaction, 5 to 15% of sodium chloride is added, often in combination with suitable antiseptics, such as pine oil or thymol. If a dry product is desired, the liquid may be further concentrated until a heavy sirup is obtained, from which the active amylase is precipitated quantitatively by addition of 2 to 3 volumes of cold alcohol or acetone. By comparing various concentrations of water-miscible solvents at different temperatures, Gates and Kneen[32] found that isopropyl alcohol, in concentrations of 40 to 60%, gave excellent recoveries of amylase without the necessity of working below 30°C.

Also, ammonium sulfate (about 50% saturation) is frequently added for the precipitation of active amylase. The precipitate may be dried by means of a vacuum tray drier at low temperature. Solid amylase concentrates obtained by precipitation with alcohol or ammonium sulfate, are too potent for most industrial purposes and are usually diluted to a standard potency by blending with inert substances, such as sodium chloride or sugar.

Amylase Production by *Bacillus subtilis* in Submerged Culture

In a recent patent, Smythe, Drake, and Neubeck[83] describe an entirely new process for the production of bacterial amylase, namely, a deep-culture fermentation method, similar to that used for the production of antibiotics. Strains of *B. subtilis* are propagated submerged in the medium, using large fermentors, provided with both aeration devices and mechanical agitation.

In order to obtain high yields of amylase, this process requires a medium considerably different from the classical media recommended by Boidin and Effront for the stationary method of amylase production. In addition to the usual protein sources used in the older process, such as 6% corn-steep liquor, 3% cottonseed meal, or 3% soybean meal, the medium recommended for deep-culture fermentation contains 4% starch plus 10% hominy feed or other finely divided cereal-grain products. The presence of this large amount of starchy substances makes it impossible to sterilize the medium unless the starch is first liquefied. This is accomplis by heating the medium to the gelatinization temperature of starch, and then adding a starch liquefying enzyme. After lique tion is complete, the medium is sterilized, cooled, and inocula with a culture of *B. subtilis;* 1 l of culture per 1,000 gal of medi is sufficient.

The temperature of the fermentor is controlled at 30°C to 40 during the growth period and aeration is at the rate of more tha volume per volume of medium per minute. After 24 to 48 hours incubation, maximal amylase yields are obtained. Filtration a further treatment are identical with those for the stationary met described previously.

This deep-culture process is of considerable interest, since had been previously reported that *B. subtilis* forms little or amylase when cultivated in a submerged medium. The process also be used for the production of bacterial protease according the claims of the inventors. It is known that at least three ma facturers are currently producing bacterial enzymes by the s merged-culture process.

Lockwood[52a] has pointed out that culture variability is a midable problem in the submerged-culture process. If a vari occurs in still cultures, it does not influence other cultures in culture vessel. In agitated-aerated cultures, the variant will grow the original culture throughout the tank and may repl it entirely in a few hours. Such rapid shifts in population are readily detected in small-scale control laboratory operations. Th fore, use of a culture that has developed a large masked variat during laboratory culturing may mean serious loss in the plant.

Other Methods For Producing *B. subtilis* Amylase

Bacterial amylase may be prepared also by a technique sim to that described for mold-bran production in the preceding ch ter. According to Beckord, Kneen, and Lewis,[5] wheat bran moistened with 1.5 to 2.5 parts of a dilute phosphate buffer, giv the moist bran an initial pH of 6.0. It is spread in thin layers a after steam sterilization and cooling, is inoculated with a liq culture of *B. subtilis.* Due to the large surface, rapid growth oc and after incubation for 48 hours at 37°C, maximum yields obtained. Certain strains of *B. subtilis* give much higher yie than others; therefore, a strain adapted to this process must ed. The same type of general equipment that is used for the mmercial production of mold bran is suitable also for this proc- s. After completion of growth, the bran culture can be dried pidly by means of warm air without loss in activity. The dried lture may be used as such, or the active enzyme may be extracted th water. A clear enzyme solution can be obtained by addition 0.5% calcium chloride to the aqueous extract, followed by tration.

Using a special strain of *B. subtilis* of the high saccharifying pe, a bran culture was obtained that, when dried, compared vorably with malt as a saccharifying agent. Especially in the esence of calcium ions, this conversion agent was considerably ore heat stable than malt and thus could be used for conversion higher temperatures. Maltose was the main breakdown product, nd the enzyme had its optimum pH at 6.6 to 7.0. To the writer's nowledge, this process has not yet been used commercially, but ndoubtedly offers possibilities for special purposes.

In a patent, issued to Christensen,[19] a method is described for ncreasing the diastatic power of barley malt or soybeans. By the ddition of an equal volume of water and subsequent inoculation f the moist product with a culture of *B. subtilis*, bacterial amylase produced in addition to the original plant amylase. After 24 48 hours of incubation, the culture may be inoculated with an mylase-producing mold, such as *Aspergillus oryzae*, *Rhizopus* or *ucor* strains, which, after another 24 hours of incubation, adds a old amylase to the mixture. In order to preserve the original nylolytic activity of the malt, no sterilization is possible and it is oubtful that such a complicated process would give consistently eliable results under nonsterile conditions. The basic principle combining amylases of different origin has considerable merit, nce often a more rapid and more complete starch digestion is tained by the combined activities of two or more amylases of fferent origin than by any amylase alone.

OMMERCIAL PRODUCTION OF BACTERIAL AMYLASE WITH *B. diastaticus*

In Russia, bacterial amylase is apparently produced com- ercially by utilizing an obligate aerobic thermophilic spore- rming microorganism, classified as *B. diastaticus*.[39] The strain of e organism used in this process is not available in the United

States at present. Growth occurs in media of strictly neutral
at temperatures between 37° and 70°C, with an optimum at 60

Following is a brief description of the commercial process as
tained from the Russian literature. The medium consists o
potato decoction. Five kg of cleaned, chopped potatoes and 10
of calcium carbonate are used per 100 l of water. The suspens
is autoclaved for 30 minutes at 22 psi pressure, then cooled to 65
and the supernatant liquor is decanted into the culture vessel. T
is a coated, glass-lined or tinned steel tank, provided with
for maintaining a temperature of 60°C. Heavy metals, such
copper and nickel, as well as sulfates and chlorides, are quite
hibitory at the high temperature necessary for growth. A spa
system, consisting of pipes with small openings, covers the bot
of the tank and is used for aeration with purified air. By usin
6 to 8% inoculum, full potency is reached in as little as 4 to 5 ho
after inoculation, during which period, the culture is thoroug
aerated. There is only a slight cloudiness evident during gro
and no development of any odor. Contamination, if it occ
sharply reduces final potency and may usually be detected by
presence of large rods with terminal spores among the thin
of *B. diastaticus*. Very simple equipment is used in the process.
impression is gained that the final fermentation is done u
aseptic, but not necessarily sterile, conditions and that the med
from the autoclave is not resterilized in the culture vessel befor
oculation. The inoculum, however, is cultivated under sterile
ditions in bottles which are provided with porous candle aerat
The inoculum bottles, in turn, are inoculated from static cult
in 5% potato decoction. The organism is maintained on a nutr
agar, consisting of 0.5% peptone, 0.1% calcium carbonate,
20% potato extract.

Without previous filtration, the culture is concentrated u
slight vacuum at 75°C or lower. The concentrate may be drie
70°C or the activity may be precipitated with ammonium sul
(60% saturation), yielding dry products of high activity.
enzyme preparation ("superbiolase") obtained is used as a desi
agent and for other industrial purposes. Its properties, as fa
can be checked, are quite similar to those of commercial bact
amylase produced by *B. subtilis*. The enzyme is exceptionally
stable and desizing temperatures of 80° to 85°C are recommend

RIFICATION AND CRYSTALLIZATION OF BACTERIAL AMYLASE

. Commercial bacterial amylase, apart from the adjuncts added preservatives or diluents, is seldom a pure product. Although ually bacterial debris is removed, it still contains the soluble comnents and breakdown products of the nutrient medium. It also ntains enzymes other than amylases; among these are usually oteolytic enzymes.

Low-molecular-weight products can be removed by dialysis cellophane bags and considerable purification can be achieved conventional methods of repeated fractional precipitation with ohol or acetone at low temperatures or by ammonium sulfate ecipitation.

By applying these methods Di Carlo and Redfern[23] obtained a ghly purified enzyme preparation with nine hundred times the tency of the original culture of *B. subtilis.* The enzyme conined 5.3% ash, 12.1% nitrogen, and 0.05% inositol. It was intivated by ficin, which indicates that the enzyme is a protein; the trogen content also suggests this. Meyer, Fuld, and Bernfeld[59] cceeded in crystallizing bacterial amylase. Although commercially ch highly purified enzyme preparations are unnecessary, they are great importance for a systematic study of their properties.

CTIVATORS AND INHIBITORS

According to Di Carlo and Redfern,[23] there is no indication r the necessity of the presence of a coenzyme for the action of cterial amylase. Studies of inactivation by iodine, silver ions, d p-Cl-Hg-C_6H_4-COOH, with subsequent reactivation by cysteine hydrogen cyanide, indicated the presence of essential -SH groups. xidizing agents, such as potassium permanganate, potassium feryanide, iodine and quinones, and heavy metal ions, such as those silver, mercury, lead, copper, zinc, and iron, have a detrimental ect and markedly inhibit the action of bacterial amylase. Other hibitory substances are maleic acid, mono-iodoacetic acid, hydrae, phenylhydrazine, semicarbazide, nitrites, oxalates, and fluides. Other cell and enzyme poisons, such as azide, salicylate, iosulfate, cyanide, sulfide, and cysteine have no effect. Exposure a pH of less than 4 or higher than 9 causes rapid inactivation of enzyme.

INDUSTRIAL IMPORTANCE OF BACTERIAL AMYLASE

Properties of Bacterial Amylase. As pointed out b commercial bacterial amylase is mainly a starch-liquefying starch-dextrinizing enzyme. Within a few minutes after add of the enzyme to a 5% gelatinized starch paste, liquefactio comes evident and progresses rapidly to completion. Soon complete liquefaction, the degraded starch no longer gives a color with iodine solution. From there on, the breakdown rea slows down considerably, but does not stop until subst amounts of reducing sugars are formed, among which gl maltose, and an unidentified fermentable trisaccharide are alent.[82] The degree of ultimate starch breakdown depen the enzyme concentration and the temperature; it is maximu 55°C and a relatively high enzyme concentration. However, under such optimal conditions, the breakdown is never com and 15 to 20% of unfermentable dextrins remain behind. Bac amylase differs from the amylases of fungi, malt, saliva, and creas in that it produces less saccharification for the same lique power.

The enzyme has its optimum activity at a pH of 6.5 to 7. (at low temperatures) has a pH range for its activity betwee and 9.0. The optimum temperature for starch digestion is at Slow inactivation becomes evident at temperatures higher 60°C. The enzyme can be employed successfully at such an ele temperature as 75°C for rapid starch digestion. Even at 100° neutral pH, its short lived action is sufficient to accomplish com liquefaction when added to a boiling starch solution.

The presence of calcium ions furnished, for example, by calcium chloride solution, markedly protects bacterial am against thermal destruction. The presence of starch also favorable influence. The outstanding heat stability of bac amylase, which is far better than that of fungal or pancreatic lase, has drawn attention to this enzyme for an increasing nu of industrial applications involving starch breakdown.[83]

Industrial Applications of Bacterial Amylase

One of the major applications of bacterial amylase is textile industry for the removal of starch sizing from the w cloth and for the preparation of sizing agents. Fiber thread,

the warp, is subjected to considerable linear strain and
ion during the weaving process, causing constant danger of
ing. In order to reduce this danger, the thread is usually
orced first by treating it with a mixture of adhesive and lu-
nt which, on drying, strengthens the thread considerably, yet
it pliable. Although there are many sizing agents used for
purpose, starch has always been one of the most widely used
cheapest one, especially on cotton, rayon, and numerous mixed
.

To give good adhesion and penetration, the starch must be
ified by a process involving a slight breakdown of the starch
cules, which causes a sharp reduction in viscosity and forma-
of dextrins. Modified starches may be prepared by a mild
ment with acids by the starch manufacturer. Often, however,
textile mill does its own modification by an enzyme treatment
e cooked starch.[39] A more uniform, better-penetrating product
tained by enzymic modification and no chemicals have to be
d which might deteriorate the yarn. Bacterial amylase is the
erred enzyme for such a modification because of the desired
ted breakdown it produces and its high-temperature activity,
h makes it possible to modify the starch without appreciable
ing immediately after gelatinization by heating.

After the weaving process is completed, the starch present in
cloth must be removed, since it would seriously interfere with
equent processing. This is done almost exclusively by enzymic
tment, the purpose of which is to bring the starch into a soluble
which can easily be washed out with water. Bacterial amylase,
quid or solid form, is at present most widely used. One of the
lest processes to accomplish desizing is to pass the cloth through
lute enzyme solution maintained at a temperature of 50° to
, during which the cloth picks up about its own weight of en-
solution. The wet cloth is then stored for several hours in
during which time the starch is converted to soluble dextrins
ater-soluble lower carbohydrates which may be removed easily
bsequent washing. Due to its fast action at high temperatures,
htly higher enzyme concentration reduces the necessary hold-
ime to a few minutes. This has opened the way to the intro-
on of a continuous desizing process in the textile industry.

Other important industrial applications for bacterial amylase
n the preparation of sizing pastes from starch for paper coat-

ings.[18,21] Reproducible, uniform results and the absence of l molecular carbohydrates are prerequisites for such sizings. T enzyme has also found extensive application for the liquefact of heavy starch pastes which form inevitably when starchy produ in high concentration must be heated, as is the case in corn-sir and chocolate-sirup manufacture,[4,100] in microbiological solv production from cooked starches or grains, and in many ot processes. Because of its extreme heat stability, the enzyme m be added during heating of the starch suspension, just bef gelatinization occurs. Liquefaction occurs as soon as the sta becomes gelatinized, thus preventing serious thickening of the ma

In the brewing industry, especially in European countr bacterial amylase has found use as an aid to the often low diast power of malt and for starch conversion of adjuncts, such as c or rice. It is also used, with proteases, for the manufacture o highly dextrinous, low-fermentable adjunct, prepared from wh and sometimes employed in the brewing industry.[91,92]

MICROBIAL PROTEASES

Proteolytic Enzymes

Proteolytic enzymes are excreted by a wide variety of mi organisms. This can be demonstrated by the simple techniqu streaking pure cultures of such organisms on a suitable solidi nutrient gelatin medium. A zone of liquefaction, observed aro the colonies formed, indicates marked proteolytic activity. A of the many bacterial species which give pronounced liquefact zones are: *Pseudomonas* species, such as *Ps. putida* and *Ps. aer nosa; Bacillus* species, such as *B. subtilis;* many sporula anaerobes, such as *Cl. sporogenes, Cl. histolyticum,* and *perfringens:* and divergent bacterial species, such as *Ser marcescens, Proteus vulgaris, B. prodigiosus, B. proteus,* and *pruni.* Among the many mold species giving liquefaction *Aspergillus niger, A. oryzae, A. flavus,* and *Penicillium roquefo*

Microbial protease is seldom one single proteolytic enz but consists of a system of proteolytic enzymes (proteases) wh include proteinases or enzymes capable of breaking down the tact protein molecule into large fragments (proteoses and pepton and peptidases (polypeptidases and dipeptidases) or enzymes wh attack only the more or less degraded protein molecules and br

m down to amino acids. There are two kinds of polypeptidases,
which attacks the peptide linkage at a point nearest the amino
l of the molecule (amino-polypeptidase) and another which
ıcks at a point nearest the carboxyl end (carboxy-polypeptidase).
the combined action of these enzyme systems, the protein mole-
e is broken down completely into its amino acid components.
crobial proteinases are usually excreted directly into the medium,
ereas peptidases are often endocellular enzymes and enter the
dium only after autolysis of the cell has taken place.

Our knowledge of the complicated mixture of proteolytic
zymes present in microbial protease liquors is still very limited.
successful separation of such enzymes has been obtained. In
neral, they form a link between the plant proteinases, such as
pain and ficin, and the tryptic enzymes of animal origin. With
exceptions, industrial interest in proteases is aimed at bringing
out the initial stages of protein breakdown; for this reason, their
oteinase activity is more important than their peptidase activity.

Bacterial Protease

As far as is known to the author, commercial production of
cterial protease is done exclusively with organisms of the *B.
btilis* group, under conditions very similar to those described for
e production of bacterial amylase.

In general, *B. subtilis* produces both amylolytic and proteo-
ic enzymes. However, the ratio in which these enzyme systems
e produced may vary considerably with the strain used, the com-
sition of the medium, the pH, the temperature, and the degree
aeration. For this reason, the strain of *B. subtilis* used for
mmercial production of protease is selected only after a thorough
dy of protease yields from a large number of strains tested and
not the same strain as used for amylase production. The medium
ed for protease production is also quite different. Contrary to
pectation, it was found that addition to the medium of carbo-
drate, such as sugar or starch, markedly increased protease yields
d often suppressed amylase excretion. In a recent study of the
t media for protease production by *B. subtilis*, Kline, MacDon-
l, and Lineweaver[47] came to the conclusion that a maximum
ld is obtained when the medium contains 0.75 to 1.0% protein
m such sources as soybean meal, peanut meal, or asparagus butt

juice and, in addition, 6% carbohydrate, such as glucose, star
molasses, and salts of calcium and phosphorus equivale
0.025% calcium and 0.05% phosphorus pentoxide. Part of t
ganic nitrogen can be replaced by inorganic nitrogen w
sacrifice in yield. The favorable influence of calcium ions o
production of bacterial protease is also stressed by other i
gators.[32,34,58]

Other typical media recommended for protease productio
those of Ramon, Richou, and Ramon[76] consisting of 8%
bran, 0.4% dried brewers' yeast and 0.6% dried malt extra
Wallerstein[95] using 12° Balling malt extract obtained by m
barley malt with addition of autolyzed yeast to bring the p
content up to 1.2 to 1.5%; or of Boidin and Effront[16] which c
of soybean flour, peanut or linseed cake to give a protein conc
tion of 0.6 to 2.5%, plus 2 to 5% carbohydrate in the form of
or starch, and 0.5 to 1% calcium or magnesium sulfate.

The equipment and technique for commercial producti
bacterial protease is identical with that used for bacterial a
production. The initial pH of the sterilized medium is 6.5
and incubation of the culture is at 37°C in shallow layers
sterile air traveling over the trays for better aeration. O
activity is usually reached in 3 to 5 days. A considerable d
potency occurs when incubation is continued too long, esp
when the pH of the culture is high.

In general, bacterial protease is less stable than bacteria
lase and considerable care has to be exercised in the preparat
a commercial protease. The culture filtrate, obtained fro
harvested culture by the same technique as described for a
production, is adjusted to neutral pH and concentrated
reduced pressures at temperatures lower than 40°C. By ad
of 2 to 3 volumes of alcohol or acetone, or 1 volume of sat
ammonium sulfate, all the active enzyme can be precipitated
precipitate is separated from the mother liquor by filtrati
centrifugation and is air dried at low temperature in vacuum
dryers. For special purposes, the concentrate may be absorb
certain inert materials, such as sawdust, and dried at low te
ture. Once bacterial protease is in dry form, it is reasonably

As pointed out before, commercial bacterial protease pr
tions can be expected to contain considerable amylase activi
vice versa. Although it is possible, by selective adsorption me

arate the two enzyme systems to some degree, this step is ally not practiced. Usually the presence of these additional e systems does not interfere with the activity of the main e and often their additional action is desirable.

The final form in which proteases are marketed depends dly on their intended use. One of the main applications is leather industry for "bating" hides. The aim of this bating s is to modify slightly certain constituents of the hide by nic digestion, for giving the resulting leather a finer grain and re, more pliability, and a far better general quality than is ned when the bating process is omitted. The opinions as to hemical changes which occur during bating differ widely. ever, a marked physical change takes place; this is apparent considerable decrease in the swelling of the wet skins. For ries, and long before the enzymic nature of the process was rstood, this digestion was done by soaking the hides for various hs of time in suspensions of dog or fowl manures.

Proteolytic enzymes, at first pancreatic preparations, but at nt also bacterial or mold proteases, have taken over the ions of the dungs. By using standardized enzyme preparations, langer of overbating, inherent in the old process and often ting in serious damage to the leather stock, is almost entirely nated. Commercial bates contain 1 to 20% enzyme prepara- depending on the potency, with the remainder being inert er, usually sawdust and ammonium salts. Since the bating ss follows the dehairing of the hides by soaking in a lime solu- it is obvious that the lime-treated skins, even after repeated ngs with water or dilute acid, are still very alkaline. This pH might cause a complete destruction of the protease, before tion is completed, but the ammonium salts tend to offset this alkalinity by acting as buffer and deliming agent. The bating is usually in the pH range of 8.0 to 8.6 and the temperature ary between 25° and 35°C. Bacterial protease, because of its d activity at relatively high pH (approximate pH range of ty is 6.0 to 9.0), is very suitable as a bating enzyme and ex- ungal protease and plant proteases whose activities do not d far above a pH of 8.0. However, the optimum pH for ial protease is 7.0 to 7.5, depending, to some extent, on the f substrate; its optimum temperature is 37°C.

nother application of microbial protease is in the textile

industry for the removal of proteinaceous sizings, such as g
or casein, which are often used as fortifying agents in the we
of silk or rayon. It is also used in liberating the silk fiber
the naturally occurring proteinaceous material (sericin) in w
the fiber is embedded. Other interesting industrial uses for
enzymes are for the recovery of silver from used photographic
for deproteinizing rubber, for the digestion of fish livers,
liberating the fish oil,[50] as a spot remover in the dry cleaning
try, and for tenderizing meat. For more detailed informatio
technical application of enzymes the papers by Gale[50-51] and p
of Triggs[86] and Wallerstein[87] may be consulted. Accordi
Lockwood,[52a] the monetary value of bacterial protease mar
exceeds $1,000,000 annually.

Fungal Protease

As has been pointed out previously, proteolytic enzyme
produced by a wide variety of fungi, including, among the
known, strains of *Aspergillus niger, A. flavus-oryzae, A. paras*
A. tamarii, A. ochraceus, A. fumigatus, A. wentii, A. effusus, P
lium roqueforti, and the mushroom fungi *Psalliota campest*
Cantharellus cibarius. Bernhauer and Knobloch[9] give detaile
on the many types of proteolytic enzymes produced by fungi.

As a rule, there is a remarkable difference in proteoly
tivity between strains of the same species of fungi, even a
strains which seem morphologically alike. Therefore, a co
able amount of screening must precede the choice of a su
strain for commercial production of fungal protease. Accord
Oshima and Church,[63] and also Ayers and Tobie,[2] the scre
may be done by cultivating the strains on such media as skim
agar or soy-milk-agar. The strains which rapidly hydrolyze
protein substrates and produce a large clear halo aroun
colonies are also usually very active protease producers when
vated under the proper technical conditions.

In the genus *Aspergillus*, there is a large proportion o
active protease producers, especially in the intermediate str
the *A. flavus-oryzae* group. It may be recalled that this grou
contains the most active amylase-producing strains. Accord
Oshima and Church[63] this group contains strains which p
high protease, but poor amylase yields; other strains are

ease, but good amylase producers; and again other strains
duce both enzymes in high yields. By using different strains,
may, therefore, obtain different types of enzyme mixture. The
ce of a suitable strain is also determined, to a certain extent,
he use for which the enzyme is intended. For instance, in the
lproofing of beer, it is desirable that the protease is active in a
range of 4 to 5; for bating of hides, however, an enzyme active
pH range of 8 to 9 is required. As early as 1918, Waksman[91]
ited out that most fungal proteases have an exceptionally wide
range of activity, much wider than that of animal or bacterial
eases. For instance, Berger[7,8] found that protease of *A. para-*
us was just as active at pH 5.5 as at pH 7.0 and that protease
l. oryzae, with an optimum at 7.0, still exhibited 40% of its max-
m activity at pH 4.3. This is in good agreement with an earlier
rt by Oshima,[61] who found this enzyme active in the pH range
.5 to 9.0. Whereas most fungal proteases have a pH optimum
und neutrality, some exhibit maximum activity at pH 4 to 5.[95]

MMERCIAL PRODUCTION OF FUNGAL PROTEASE

For commercial production of fungal protease, a variety of se-
ed strains of *A. flavus, A. oryzae, A. wentii, Mucor delemar,*
ylomyces rouxii, and others are used.[95] The medium on which
e fungi are cultivated is usually wheat bran,[50,85,92] soybean
e,[92] alfalfa meal,[92] broken grain, middlings, brewers' grain,[95]
t, or mixtures of these compounds. Methods of preparing
media, the technique of cultivation, and the equipment used
identical with those for the production of fungal amylase by the
d-bran process (see Chapter 3) and, therefore, need no further
ussion. Layers of 1 to 2 in. are used if the culture remains un-
urbed and 2 or 3 ft if agitated.[95] Cultivation is continued for
5 days in a moist atmosphere at 30° to 40°C.

As soon as abundant spore formation occurs, all proteolytic
mes are no longer intracellular but can be recovered quantita-
ly from the medium.[64,65] Harvesting must then take place since
nsiderable decrease in enzyme potency is observed on further
bation. The mold bran is dried at low temperatures or the
e protease may be recovered by extraction with water or buffer
H 6.5. The extract is concentrated under vacuum at tempera-
s below 40°C and the active enzyme may be precipitated by

addition of 3 volumes of alcohol or acetone, or with ammonium sulfate, by a method similar to that described for bacterial protease.

RECENT INVESTIGATIONS OF FUNGAL PROTEINASE PRODUCTION

In 1950, Maxwell[53a] reported a laboratory investigation made for the purpose of selecting suitable strains of *A. oryzae* and satisfactory conditions for large-scale production of proteinase for fellmongering wool from sheepskins. She reported that wheat bran containing 60% moisture was the best medium. The best conditions were sterilization by steaming for 3 hours and, after inoculation, incubation in 2 cm layers at 28°C for 89 hours. Best enzyme production was obtained with sparsely-growing, heavily-sporulating cultures obtained by drying near the termination of the growth period.

Considerable interest has developed recently in submerged culturing of molds for proteinase production. Dworschack, Koepsell and Lagoda[24a] surveyed four hundred ninety-one strains of the *A. flavus-oryzae* group and one hundred thirty-one strains of other *Aspergilli* in shake-flask cultures on 2% corn meal–1% soybean meal–0.5% calcium carbonate medium. Eighty strains of the *A. flavus-oryzae* group produced sufficient proteinase to warrant further study. Certain strains were found to be far superior and the enzyme from individual mold strains varied in ability to attack the various proteinaceous substrates tested, including gelatin, casein, and milk. Ability to hydrolyze gelatin appeared frequently; milk-clotting activity was rare. High amylase activity was found very infrequently in the cultures tested and was never associated with high proteinase activity. Culture filtrates from high-yielding strains were concentrated by vacuum evaporation and then dried to powder by lyophilization. The resulting solid enzyme preparations had higher casein-digesting ability than twelve commercial proteinase preparations tested for comparison. It was concluded that proteinase production by submerged cultivation of certain strains of the *A. flavus-oryzae* group is feasible. By selection of particular mold strains and culture conditions, it may be possible to favor some types of proteolytic activity over others.

USES OF FUNGAL PROTEASES

Fungal protease has optimal activity at 50°C,[64] and is used for the same purposes as bacterial protease. When digestion at a low pH is required, it has advantages over bacterial protease. In Oriental countries, for many centuries, fungal proteolytic enzymes have played the main role in the manufacture of such food products as soy sauce, tamari sauce, miso and tofu. The last two products are used as breakfast foods, the former being a soy-cereal paste and the latter a cheeselike product. These are prepared from cooked or steamed soybeans, often mixed with roasted wheat or rice, by mixing with *koji* (or starter) prepared on similar media inoculated with selected strains of *A. flavus-oryzae* or *A. tamarii.* During incubation in thin layers on trays, under controlled humidity and temperature, the beans become covered with mold mycelium. At the time of maximum enzyme production, brine is added and the enzymic digestion is allowed to proceed for a considerable length of time, often several months. Detailed descriptions of such processes have appeared in the literature.[20,45,60,77]

Mold proteases are also important components of the so-called "chillproofing agents" used in the brewing industry for clarification and maturing of beer and ale and for correcting hazes which often develop in beer during storage. Such turbidities, thought to be due to protein-tannin complexes, often develop under adverse conditions of temperature, especially under repeated agitation and cooling. Addition of proteolytic enzymes, active at relatively low pH, causes a slight digestion of these proteins and thus prevents the formation of insoluble complexes with tannin. Introduced in 1910 by Wallerstein,[93] modern chillproofing agents are usually mixtures of animal, plant, and microbial proteases, containing pepsin, papain, bacterial and fungal protease.[95] According to Wallerstein,[99] a tannase of fungal origin, added to such a proteolytic enzyme mixture, gives an even greater degree of stability by causing digestion of both components of the complex.

MICROBIAL PECTOLYTIC ENZYMES

Pectin-hydrolyzing enzymes have found considerable industrial application for the pretreatment of fruit juices and related liquids as an aid in their clarification. Commercial pectolytic enzymes are usually known by their trade names, "Pectinols" or "Filtragols." Little technical information is available on the manufacture of such enzyme preparations, but it is likely that they are obtained from mold strains of *Penicillium* or *Aspergillus* species. The Pectinols are seldom pure enzyme preparations and often contain more tha 90% inert matter, such as sugar, apparently as a diluent.[illegible] Th active part contains at least two types of pectin-decomposin enzymes. More detailed discussion of the pectic enzymes, with regard to nomenclature, occurrence, methods of determination, purification, specificity, and mode of action, may be found in th reviews by Phaff and Joslyn,[73] Kertesz,[46] and Lineweaver an Jansen.[51]

The nomenclature of pectolytic enzymes has been somewha confused and the two most important ones are known by severa names.

The enzyme which hydrolyzes the methoxyl groups in th soluble pectin molecule, with formation of methanol and polygalacturonic acid, has been variously designated as pectinesterase, pectin methylesterase, pectase, pectin demethoxylase, pectin methoxylase and pectolipase.[51,54] The first two names seem most suitable for th enzyme that deesterifies pectin, since they indicate the nature of th enzyme action, while pectase is the oldest name. This enzyme wil be referred to in the following discussion as pectinesterase.

The enzyme which hydrolyzes soluble polygalacturonic aci to monogalacturonic acid has been called polygalacturonase pectin-polygalacturonase, pectinase, and pectolase.[46,51] Although th older name, pectinase, is frequently used to designate this enzyme it has also been used to designate pectolytic enzyme mixtures. A the first two names are more descriptive of the enzyme action, th term polygalacturonase will be employed here.

A third pectolytic enzyme, protopectinase, which hydrolyzes th insoluble protopectin of the cell wall of the plant into solubl pectin, is also present.[22] However, the existence of a separat enzyme for this reaction is not proved beyond doubt and protopectinase activity of commercial pectinols may be exerted by thei polygalacturonase.[26]

Pectinesterase and polygalacturonase can be separated by fractional adsorption on cationic-exchange resins at pH 6.3, or b destruction of the pectinesterase activity at pH 0.6 and 25°C for 2 minutes.[41] Besides pectolytic enzymes, commercial pectinols contain also diastatic enzymes, invertase and maltase.[29]

Pectolytic enzymes are produced by several bacterial species including *B. carotovorus,*[44] *B. subtilis,*[28] *B. mesentericus* var. *fuscus,*[27] *Cl. felsineum*, *Cl. acetoethylicum,*[3] *Ps. marginalis*[68] when cu tivated under proper conditions. Very little is known about the properties of such bacterial enzymes. They may play an important role in the retting of flax.

Of much more industrial interest are the pectolytic enzymes of fungi. Many *Aspergillus* species, such as certain strains of *A. niger,*[3,22,101,102] *A. flavus,*[101,102] *A. oryzae,*[75,91,92] *A. fumigatus,*[101,102] *A. parasiticus,*[101,102] and *A. wentii*[101,102] are good producers of such enzymes. *Penicillium* species, such as *P. glaucum,*[101,102] *P. ehrlichii,*[26] and *P. chrysogenum,*[72] *Rhizopus* species, such as *R. tritici,*[22,35,101,102] and *R. nigricans,*[75,101,102] *Monila fructigena,*[57] *Botrytis cinerea,*[3,22,28,57,75] *Fusarium chromiophthoron,*[57] *Fusarium fructigenum,*[57] and *Mucor* species[75] have all been reported to excrete active pectolytic enzymes.

It has been found that production of pectolytic enzymes by molds is usually greatly stimulated by the presence of pectin or pectin-containing compounds in the nutrient medium.[3,23,35] Pectic acid, D-galacturonic acid, mucic acid, L-galacturonic acid, and galacturonic-acid-containing gums, such as gum tragacanth, also stimulate production of pectolytic enzymes with certain mold strains.[72] It seems reasonable, therefore, to assume that pectin- or galacturonic-acid-containing compounds are probably one of the main carbon sources in the industrial production of such enzymes. According to Lockwood,[52a] the commercial pectin-hydrolyzing enzymes are produced by *Penicillia* grown on pectin-containing substrates, such as sugar-beet cossets.

As a nitrogen source ammonium sulfate,[72] yeast or malt extract,[28] potato decoction, asparagin, peptone, ammonium tartrate,[28] and gelatin or casein[13] have been recommended. According to Fernando,[28] the best nitrogen sources (for *Botrytis* strains) are those which cause a pH shift to the alkaline side. As a result of the action of the excreted enzymes on pectin, the pH drops considerably due to the liberation of free carboxyl groups.[75]

The ratio of the two pectolytic enzymes in the culture filtrates varies greatly with the fungus used.[73] Often the rate of pectin breakdown by commercial preparations is limited by their low pectinesterase content. Jansen and MacDonnell[41] were able to increase the activity seven times by the addition of extra plant pectinesterase to such preparations. Phaff and Joslyn[73] suggest, therefore, the combination, in proper proportion, of the enzymes of two fungi, one rich in pectinesterase, the other rich polygalacturonase.

There is also a great variation in the excretion of pecto enzymes by different strains of the same mold species,[71] even w cultivated on the same medium under identical conditions. choice of a high-yielding strain is, therefore, of prime importa

The pectolytic-enzyme system is partially excreted into medium and partially remains in the cell.[28] Certain mold spe such as *R. chinensis* and *R. microsporus*, excrete almost all enzymes into the culture medium.[55] In order to obtain the en from the mold mycelium, this must be dried, ground, and extracted with water. The enzyme system may be obtained in form from such a mycelium extract, or from the culture medium precipitation with 3 to 4 volumes of alcohol or acetone.[25]

The optimum pH range for fungal pectinesterase activit 3.0 to 4.5; that of fungal polygalacturonase activity is between and 4.5. Optimum temperature for both enzymes varies betw 37° and 55°C.[29,71] At neutral pH, or at temperature of 75°C. enzymes are rapidly destroyed. Bacterial polygalacturonase h much higher pH optimum, close to neutrality,[25,63] making it suitable for clarification of acid fruit juices.

The first commercial application of pectolytic enzymes wa the pretreatment of fruit juices, wines, vinegars, sirups and je as a clarification aid for such products.

Most fruit juices, obtained by expressing fresh fruits, as apples, apricots, oranges, and grapes, are quite turbid due t presence of finely dispersed colloidal matter. The market of certain of these, such as apple cider, is much higher when juice is sparkling clear.[53] However, filtration to a clear cide almost impossible, unless the colloidal matter responsible for turbidity is first eliminated or coagulated. Since the food val well as the original flavor of fresh fruit must be retained, the of processing aids is rather limited and experience has shown a proper enzyme treatment gives the best results. Cloudines fruit juices is greatly increased by the presence of pectins pectinlike substances which act as protective colloids for suspen particles. Moreover, the presence of pectins gives the juice a viscosity which also tends to stabilize the colloidal suspensio e pectins are broken down by enzymes, the colloidal particles their stability, and a flocculent precipitate is formed which can asily removed by filtration, yielding a clear fruit juice.

Another recent application of pectolytic enzymes is the re- al of pectins from fruit juices which are to be concentrated. example, an apple juice which is concentrated without removal he pectins will jell. Removal of pectins is necessary in such entrated fruit juices to keep them liquid.

In several cases, it has been found advantageous to add pecto- enzymes to crushed fruits before expressing the juice. Break- n of the pectin at this stage often increases the yield of juice siderably and facilitates subsequent expressing.[36] Addition of olytic enzymes to crushed grapes results in greater ease of sing and greater yield of juice.[36,106] Treatment of certain grape es with pectolytic enzymes prior to fermentation has been ribed; the resultant wines clarify much sooner than those le from untreated grape juice.[19,36] (See Chapter 7 of Volume I.)

Another possible application for pectolytic enzymes is in the duction of D-galacturonic acid.[78] Galacturonic acid is a use- compound for a number of organic syntheses, including that itamin C. Less valuable pectins, such as those from sugar beets itrus waste products, might be used for such a process.[25,38,40,78]

The application of tomato pectinesterase for the preparation of galacturonic acid for use as low-solids gels or pectinate coatings been patented.[104] Some glucosidic degradation by polygalac- onase in the tomato-enzyme preparation can be obviated by g citrus-peel pectinesterase[66] which is free of polygalacturonase. h low-methoxyl pectins are too sensitive to precipitation by ium to be used in the practical preparation of low-solids gels, they form very viscous solutions and may have useful applica- s, e.g., as paper coatings and agar substitutes.[67]

The use of commercial pectolytic enzymes to determine "pectic me soluble substances" of foods and food products has been mmended.[40] However, since the commercial pectolytic-enzyme arations contain several other polysaccharide-hydrolyzing mes, such as amylase and inulase,[29,52] the use of purified galacturonase in combination with a deesterifying agent could nore desirable for this purpose, if available.[52]

INVERTASE

Invertase (sucrase or saccharase) is the enzyme
for the biochemical hydrolysis of sucrose into glucose a
The enzyme also hydrolyzes certain tri- and tetrasaccha
moving either the fructose or the glucose end of th
Invertase occurs widely in many animal and plant tis
as in a great variety of microorganisms, such as num
species, filamentous fungi (*A. oryzae, A. niger, P. gla*
bacteria (*E. coli, Thermobact. mobile*).

As a rule, fungi contain less invertase than yeasts
invertase differs fundamentally from yeast invertase.
vertase is a fructosidase (β-h-fructosidase, fructofuran
tacking the fructose end of the molecule, whereas fung
is a glucosidase (α-h-glucosidoinvertase, α-glucopy
attacking the glucose end of the molecule. This explai
types attack sucrose, but differ in their behavior tow
tri- and tetrasaccharides. For instance, the trisacchari
(fructosylglucosylgalactoside) is split into fructose an
by yeast invertase, but is not attacked by fungal invert
the glucose is blocked by the glucosidic linkage with

Invertase is an endoenzyme and can be isolated
mechanical disruption of the cell wall or after aut
mercially, it is prepared from bakers' or brewers' yeast

The invertase content of yeasts varies widely wit
strain and with the conditions of growth. The invert
yeasts may be increased five to ten times by cul
the presence of sucrose.[96] One of the procedures for ac
this is as follows: To a 5% suspension of beer-yeast
each of diammonium and dipotassium phosphate an
magnesium sulfate are added. After adjustment of the
the yeast suspension is vigorously aerated for about 8 ho
During this period, the equivalent of 5% sucrose is adde
maintaining the pH at 4.5. Other manufacturers of yea
instead of using treated commercial bakers' or brewers'
vate a special strain of *Saccharomyces cerevisiae* of e
high invertase content at controlled pH and temperat
using sucrose as a nutrient.[97,98]

After this pretreatment, resulting in a considera
in invertase yield, the yeast is filtered and the yeast

a plasmolyzing process, followed by autolysis. Chloro-
er, ethyl acetate, or toluene is usually used as a
ing agent. The following is an example: To 100 lb of
er yeast cake 10 lb of toluene is added and the mixture
to liquefy for 3 hours. Then 12 gal water is added and
djusted to 5.8. Autolysis is allowed to proceed for 21
30°C. After completion of the autolysis, the cell debris
parated from the autolysate by centrifugation, after ad-
e pH to 4.7. The invertase, which remains in the
, may be precipitated by addition of 1.25 to 1.5 volumes
l at pH 4.7 and 0°C. Better precipitating agents, which
potency loss, are isopropyl and isobutyl alcohols[81] or
ble ethers of the dioxane type.[82] The precipitate is either
ow temperature or dissolved in 60% glycerol, ethylene
annitol, or sucrose.

rtase has its optimum activity in the pH range 3.5 to 5.5
so most stable in this range. The more the enzyme is
the more unstable it becomes. This is why most com-
nvertase preparations are not highly purified.

rtase is used in the manufacture of artificial honey and
gar, the second being much more soluble than sucrose.
n advantage in confectionery manufacturing and in the
on of liqueurs and ice creams where crystallization of
r concentrations must be avoided. A carefully standardized
preparation is usually incorporated in the formula for
hocolate-coated, soft-cream-center bonbons. The molding
ng of the bonbon is done while the content is still firm
enzyme conversion and liquefaction of the sucrose to a
d stable cream takes place later.[83] Enzymically produced
gar is also used in the paper industry as a plasticizing
Neuberg and Roberts[84] have published a detailed litera-
w on invertase.

MISCELLANEOUS MICROBIAL ENZYMES

n the last few years several other microbial enzymes
ared on the market. Few details are available as to the
methods used in producing these enzymes, but they are
ss worthy of mention.

se, an enzyme which decomposes hydrogen peroxide to oxygen and water is marketed in liquid preparations. It is us in bleaching and dyeing furs, in bleaching human hair to remo residual hydrogen peroxide, and in the manufacture of surgic gut. At least one commercial product is obtained by growi a mold in aerated-agitated culture.[52a]

Penicillinase is manufactured by aerated-agitated culturing *B. cereus* or *B. subtilis*.[52a] It is precipitated from the clarifi culture liquor with an organic precipitant, and is marketed as dry enzyme concentrate. It finds greatest use in penicillin-produ tion control where the Ford[29a] rapid assay method is used.

A new enzyme preparation was placed on the market in 19 which contains a mixture of two enzymes, streptokinase a streptodornase.[1a] The preparation is made by hemolytic strep cocci grown in aerated culture. It is used clinically in dressi wounds and burns as it disintegrates and clears away blood clo pus, and dead tissue without attack on healthy tissues. Sever production problems have been mentioned, including cultu selection, purification of the enzymes, and effect of medium co position on enzyme yields.[1a,9a,26a]

The most recent commercial enzyme is glucose-oxidase. Is marketed by at least two companies.[1b,1c] It is obtained in assoc tion with catalase by submerged culture of *A. niger*[52a] and marketed in the form of a soluble powder. A principal use of t enzyme preparation is to remove glucose from egg whites a whole eggs before drying to prevent browning and deteriorati Oxidation of the glucose by the glucose-oxidase forms glucor acid and hydrogen peroxide, the hydrogen peroxide then bei decomposed by the catalase.

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PART IV. THE PRODUCTION OF VITAMINS

PTER 5

PRODUCTION OF RIBOFLAVIN BY FERMENTATION

R. J. Hickey

INTRODUCTION

Microbiologically produced riboflavin, or lactoflavin as it is also
own, has long been available in yeast and in related preparations
association with many other vitamins, particularly those of the
omplex (see Chapter 6). The general history of riboflavin has
n widely covered in reviews, such as those of Rosenberg,[67]
opfer,[69] and Van Lanen and Tanner.[92]

Riboflavin is a unique vitamin in that it can be totally synthe-
d to very high concentrations rather rapidly by certain micro-
anisms. Reports on authentic precursors or adjuvants for
synthetic riboflavin have not appeared, but the mechanism of
mation has had recent study by Plaut.[47a] Fermentations have
n reported in which the riboflavin content of the fermented
r amounted to 1,500 to 2,000 μg per ml and higher. The organ
s involved in such processes are the *Ascomycetes, Eremothecium*
byii,[40] and *Ashbya gossypii.*[54] Fermentation riboflavin has a
d position in competition with synthetic riboflavin.

In 1948, the United States Tariff Commission reported the pro-
tion of 130,400 lb riboflavin for human, animal, and poultry

consumption. Of this, 113,000 lb were sold, valued at $7,033,2 or about $62 per pound. These figures were not broken down in production by fermentation and chemical synthesis, but it is know that a very considerable proportion of this quantity was ferment tion riboflavin. The price of riboflavin in 1953 was in the range $45 to $60 per pound.[7a]

Riboflavin is synthesized, to some degree, by many various typ of microorganisms, including bacteria, yeasts, and molds.[92] Oth organisms require riboflavin for growth and may, therefore, be us for assays.[79] The organisms which have been found to produ sufficient riboflavin to be, or to have been, of interest from a co mercial standpoint are relatively few. Those of prime importan for riboflavin formation rather than for the B-complex are not in Table 13. In this table, the relation of iron concentration riboflavin formation by the organism is also noted, since in so processes, the iron content of the medium is a factor controlling t riboflavin yield. Other bacterial genera have also been reported[91] which produce quite low levels of riboflavin.

TABLE 13. MICROORGANISMS PRODUCING CONSIDER ABLE RIBOFLAVIN AND THE EFFECT OF IRON ON TH BIOSYNTHESIS

Organism	Riboflavin in culture fluid, μg per ml	Optimum iron concentration, μg per ml	Reference
Mycobacterium smegmatis	57.5	Not critical	32
Clostridium acetobutylicum	97.0	1 to 3	34
Mycocandida riboflavina	200.0	Not critical	29a
Candida flareri	567.0	0.04 to 0.06	26
Ashbya gossypii	1760.0	Not critical	54, 56
Eremothecium ashbyii	2480.0	Not critical	40

Commercial fermentation processes for production of riboflav or riboflavin concentrates are relatively recent, having been veloped since about 1937. Aside from food yeasts, the first organis employed primarily for riboflavin production was *Clostridiu acetobutylicum*, which was used both in the United States and Japan. Following the butyl alcohol-acetone fermentation of gra or molasses media, the solvents were removed by distillation and t still residues were dried to yield products containing about 20 65 μg of riboflavin per gram of residue. Such products were of co derable value not only because they could be used profitably as a ed supplement, but also because drying the slops provided a very ood solution to a stream-pollution problem of some manufacturers f solvents.

The *Cl. acetobutylicum* fermentations of grain or lacteal products, e.g., whey, were improved during the succeeding years after it as found that by controlling the iron concentration in the media, ried residues containing riboflavin to 4,000 to 5,000 μg per g and igher could be obtained.

About 1940, it was discovered in the United States that an *scomycete, Eremothecium ashbyii,* was able to convert nutrient edia and, in particular, cheap waste products, such as residues rom the alcoholic fermentation of grains, into riboflavin solutions f 200 to 400 μg per ml or higher. The fermentation could be onducted in submerged culture in about 4 to 6 days and the iron ontent of the medium was not particularly critical to the riboflavin ield. Ordinary steel equipment was satisfactory and the resulting 'beers" were of sufficient riboflavin potency to make recovery of rystalline riboflavin practicable.

Within the last few years, improved *E. ashbyii* fermentation rocesses have been described in the patent literature. Yields of ,000 to 2,000 μg per ml or higher have been reported. Since 1946, another *Ascomycete, Ashbya gossypii,* has also been reported as being capable of producing high-riboflavin beers from cheap raw materials. Yields higher than 1,000 to 1,500 μg per ml have been reported. The *Ascomycete* processes are now assuming a fairly important position in the riboflavin-manufacturing field.

COMMERCIAL FERMENTATION PROCESSES FOR RIBOFLAVIN PRODUCTION

Clostridium Acetobutylicum Processes

The *Clostridium acetobutylicum* processes for the formation of riboflavin are anaerobic, in contrast to the *Candida, Eremothecium ashbyii,* and *Ashbya gossypii* processes which are aerobic. As for the *Candida,* appreciable riboflavin formation by *Cl. acetobutylicum* is dependent on a low iron content of the medium. The *Ascomycete* processes (*E. ashbyii* and *A. gossypii*), however, are relatively unaffected by reasonable concentrations of iron, such as might be introduced by handling in iron or steel equipment.

There are primarily three methods which have been o being used for riboflavin production by *Cl. acetobutylicum* classification is based largely on the medium, or substrate, an (a) the molasses process, (b) the grain process, and (c) the product processes. Relative yields of some of these processe indicated in Table 14.

TABLE 14. BUTANOL-ACETONE PROCESSES FO RIBOFLAVIN PRODUCTION

Medium	Riboflavin yield µg per ml	Refe
Molasses	1 to 2	[illegible]
Grain, corn	25 to 30	[illegible]
Grain, corn and rice	40 to 50	[illegible]
Whey or skim milk	20 to 50	[illegible]
Whey and xylose	70 to 97	[illegible]

Since contamination of the media with iron is a major which may limit riboflavin production by *Cl. acetobuty* various means of controlling the iron content have been st and employed. The best method, of course, is to prevent en iron into the medium in the first place. In the grain proces excess iron is removed by mechanically cleaning the grain. I milk products processes, the lacteal raw material (e.g., wh produced under conditions[88] which limit the iron content medium. Molasses generally contains considerable amounts organic matter, including iron, and fermentations usually a low-riboflavin "beer" containing about 1 to 2 µg of ribofla ml. Control of iron concentration in the medium by the 2,2′-bipyridine (α,α′-dipyridyl)[77,78] to bind it as biological available iron was unsuccessful in producing higher ribo yields in molasses media, although this method worked satisfa in grain media. The reason for the difference is not clear.

Other methods for obtaining improved riboflavin yield been reported. Yamasaki[100] added calcium carbonate or ace grain media during the first 7 hours and obtained higher Van Lanen and Tanner[98] consider this to be a practical appl of the methods of Steinberg[90,91,92] who removed trace ele in this case iron particularly, by adsorption on calcium car Legg and Beesch[44] described a process which employed 0.

% sodium sulfite or other sulfites in grain mashes for improved
ls.

Recently, Leviton,[47,48] using synthetic-type media, found that
ıddition of certain chemicals to a medium containing an exces
amount of iron would allow fermentation to proceed with a
l yield of riboflavin. Among these agents were catalase, potas-
ı iodide, sodium hydrosulfite, and yeast dialysate. Leviton
gested that hydrogen peroxide may be involved in riboflavin
ruction when appreciable iron is present. No peroxide was
nd in the beer, however, and a transient existence was postulated.
: fact that the presence of catalase is effective in promoting
ıer riboflavin yields certainly suggests the presence of a peroxide.
iton showed that iron catalyzes the decomposition of riboflavin
he presence of hydrogen peroxide. The aerobic, high-riboflavin-
ducing *Ascomycetes*, such as *Eremothecium ashbyii*, undoubtedly
duce catalase. These organisms also perform well in the presence
ufficient iron to cause low riboflavin levels in *Cl. acetobutylicum*
cesses. They yield riboflavin in excess of 1,000 μg per ml. The
ndida processes are aerobic and are very sensitive to iron. Catalase
duction would also be expected here.

E MOLASSES PROCESS

The fact that riboflavin was produced during the butyl alcohol-
tone fermentation of molasses media was described by Miner[35]
a patent in 1940. He pointed out that the dried residue of the
lasses fermentation was a good source of riboflavin and other
mbers of the B-complex. The riboflavin content of the "beer"
to 2 μg per ml.

When a high-test molasses medium is employed, following
nentation for about 72 hours at 32°C, the solvents are stripped in
ill and the residue, or "slop," is concentrated and dried. Spray
drum driers can be used. The dried product may contain about
o 60 μg of riboflavin per g, along with other B-complex vitamins.
e product was found to be quite useful and profitable as a feed
plement.

Several organisms have been described in the patent literature
use in the molasses process. An example is *Cl. saccharo-butyl-*
onicum-liquefaciens-delta.[35] Such organisms are considered by
gey[3] to be types of *Cl. acetobutylicum.*

As was previously noted, control of iron content of the me at low levels did not result in improved riboflavin yields,[17] in co trast to the findings in the grain and lacteal product fermentatio The fermentations are generally carried out in the customary st equipment.

THE GRAIN PROCESSES

The fact that acetone-butyl alcohol fermentations of gr yielded a yellow product under some conditions had been kno for many years.

About the same period that Miner[35] noted that riboflavin formed in the butyl fermentation of molasses, Yamasaki[97,98,99,100] observed that cereal grain fermentations could yield a yellow p ment which was identified as riboflavin. He obtained a Uni States Patent[101] on the process in 1942. Yamasaki's method[101] ploys *Cl. acetobutylicum* for the fermentation of 4 to 8% cer grain mashes, to which, between 0 and 7 hours after inoculation small amount of calcium carbonate or calcium acetate is add The use of calcium salts resulted in considerably increased ri flavin yields. In addition to grain mashes, Yamasaki also emplo whey and other carbohydrate media with good results.

In 1943, Arzberger[2] pointed out that one of the basic fact controlling riboflavin formation in the *Cl. acetobutylicum* ferm tation of cereal-grain mashes is the concentration of certain meta ions in the medium. In particular, Arzberger cited iron, cob nickel, copper, zinc, and lead salts as having a deleterious effect riboflavin formation if present in sufficient concentration. data show that, of the salts mentioned, those of iron and col suppress riboflavin formation at the lowest levels cited, i.e., 3.2 of the salt per l of medium. Increasing concentrations of salts the other metals, except nickel, did not strongly suppress ribofla formation until the fermentation was suppressed as indicated low yields of solvents. Nickel acetate, at about 320 mg per l, pressed the riboflavin yield without appreciably affecting solvents, but one-tenth and one-hundredth of this level did greatly affect riboflavin synthesis. It is possible that the ribofla suppressive effect of the nickel acetate at about 320 mg per l due largely to the presence of iron and cobalt impurities in nickel salt. Some of Arzberger's data are given in Table 15.

TABLE 15. EFFECT OF SOME HEAVY-METAL SALTS ON RIBOFLAVIN FORMATION IN 5% CORN MASH BY *Cl. acetobutylicum*

	Riboflavin and solvents yields[a] at the salt concentrations noted									
Salt concentration,	0		3.2		32.0		320.5		3205.0	
mg per salt added	Riboflavin	Solvent	Riboflavin	Solvent	Riboflavin	Solvent	Riboflavin	Solvent	Riboflavin	Solvent
Ferrous sulfate ($7H_2O$)	2,720	26.4	810	26.6	197	27.8	69	28.0	145	24.3
Cobaltous acetate ($4H_2O$)	3,150	29.0	620	27.7	242	27.9	64	12.8	—	—
Nickel acetate ($4H_2O$)	3,150	29.0	2,850	28.7	2,240	28.9	680	28.9	38	6.6
Cupric acetate ($1H_2O$)	2,890	28.8	3,180	31.0	2,030	31.0	41	6.7	—	—
Zinc acetate ($2H_2O$)	2,890	28.8	3,430	27.6	2,945	29.4	2,440	29.2	55	4.0
Lead acetate ($2H_2O$)	2,890	28.8	2,500	29.6	2,295	29.8	2,110	28.4	39	5.3

[a] Riboflavin yields are expressed as micrograms per gram of dried filtrate. Solvents yields are expressed as percentage of original corn, dry basis.

Source: Arzberger.[2]

Riboflavin is formed in the corn fermentation process, extent of 1 to 2 μg per ml, when operated in iron or steel ment. In glass or aluminum equipment, mashes prepared cleaned grain, low in iron, yield about 25 μg per ml or more flavin after fermentation.

Walton[88] described a modification of Arzberger's meth which mixtures of corn and rice were used in the mash inst corn alone, as cited by Arzberger. Riboflavin yields of 40 to per ml were obtained. Legg and Beesch[44] used wheat flou other flour and obtained about 26 μg riboflavin per ml. processes of both Arzberger and Walton, the grain was subje special handling, such as blowing with air and magnetic tre for removal of iron-containing substances. In both pr *n*-butyl alcohol, acetone, and ethyl alcohol were by-prod economic value. Walton cited riboflavin contents of up to μg per g in the dried filtrates.

The optimum iron concentration for maximum rib formation by *Cl. acetobutylicum* is in the range of 1 to 3 μg pe which is fifty to one hundred times as high as the optimum le the *Candida* processes.

The general fermentation procedures are typical of th the regular butyl alcohol-acetone fermentation of grain (see ter 11 of Volume I), except that excess iron contact must be av Aluminum equipment has been recommended.[8]

THE MILK-PRODUCT PROCESSES

In 1938, Yamasaki[107] found that if a whey-type medium jected to the butyl alcohol-acetone fermentation and a calciu such as the carbonate, is added during the first 7 hours of fer tion, considerable improvement in the riboflavin yield will

In 1945 and later, Meade, Pollard, and Rodgers[55,64,6 tained several patents[illegible] describing methods for rib formation by the *Cl. acetobutylicum* fermentation of lacte terials. The basis of the method was primarily control of organic nutrition of the organism during fermentation, tho supplementary use of xylose[54] also resulted in substantial in in riboflavin yield. Methods of handling were also import

Lacteal products, such as whey and skim milk, contai bers of the B-vitamin complex, including riboflavin, alon other nutrients, such as lactose and casein.[88] The riboflavin

is approximately 1.7 μg per ml. If whey is prepared in a
d commercial manner, i.e., in iron or steel vessels, the iron
may be as high as 10 or 12 μg per ml (or ppm). Whey pre-
n the laboratory by rennet treatment of skim milk was
o contain about 0.37 μg per ml iron. Meade, Pollard, and
,[33] found that the commercial high-iron whey underwent
acetobutylicum fermentation quite vigorously with good
utilization and good solvents yield, but the riboflavin yield
oor, being around 1.8 μg per ml. The low-iron whey
g per ml) was found to ferment sluggishly and incompletely,
considerable lactose unfermented after 48 hours at 38°C.
boflavin yield was about 6 μg per ml. If the total iron was
d to 1.5 to 2.0 μg per ml before sterilization, the fermenta-
as quite vigorous and complete and the riboflavin yield was
30 μg per ml.

was mentioned[33] that it was of advantage to include trace
ts of salts of strontium, tin, manganese, lithium, and zinc in
dium. The recommended concentration was 0.00003 molar.
edium was adjusted to pH 6 to 7 prior to fermentation. The
were sterilized for 15 to 20 minutes at 121°C. The process
up to 50 μg per ml of riboflavin. Some of the data are
rized in Table 16.

16. EFFECT OF IRON CONTENT OF WHEY MEDIUM
RIBOFLAVIN PRODUCTION BY *Cl. acetobutylicum*

	Concentration of Agent in Medium			
	Sugar (as lactose), %	Protein, %	Iron, μg per ml	Riboflavin μg per ml
nfermented	4.70	0.90	variable	1.7
rmented, low iron	3.20	0.80	0.37	6.0
rmented, high iron	0.53	0.74	5.00	1.8
rmented, m iron	0.71	0.74	1.5 to 2.0	20 to 30

Meade, Pollard, and Rodgers.[33]

an improved process, in 1947, Meade, Rodgers, and Pollard[34]
that the inclusion of xylose in the lacteal medium results
oved riboflavin yields from the *Cl. acetobutylicum* process.
in a concentration of 0.5 to 1% may be added, though some-
gher or lower levels are still useful. The media were also
ented with 0.15% by weight $CaCO_3$, 0.15% $Ca_3(PO_4)_2$,
er ml $ZnSO_4 \cdot 7H_2O$, 4.2 μg per ml $MnSO_4 \cdot H_2O$, and 0.01 μg

per ml *p*-aminobenzoic acid. Iron is cited at a concentration of about 1 to 2 μg per ml, though the iron range is claimed between 0.5 and 4.5 μg per ml.

Without the supplementary xylose, riboflavin yields of abou 60 to 64 μg per ml were noted, while with about 0.5 to 1.0% xylose added, riboflavin yields of 92 to 97 μg per ml were obtained. The authors felt that xylose did not act simply as another energy source Xylose can be supplied by the use of sulfite liquor, wood hydrolysates, etc.

Several related patents were issued to Pollard, Rodgers, and Meade. These correlate variable concentrations of iron in the lacteal media to the zinc content as it affects riboflavin formation,[5] to the magnesium content,[51] to the manganese content,[53] and to the ammonium content.[50] The relationship between iron content and pH is detailed in other patents,[49,65] while inoculum development is described in still another.[66]

Fungal Processes

SYNTHESIS BY YEASTS

Food Yeasts. Commercial food or bakers' yeasts contain 40 to 85 μg riboflavin per g.[92] Investigations by Massock[31] and by Van Lanen[92] of methods for improving riboflavin formation by *Saccharomyces cerevisiae* were unsuccessful. Others[43,77] have been able to bring about minor alterations, but no methods have been found which would produce riboflavin specifically in high concentration by the use of *Saccharomyces.* This is also true for *Torulopsis utilis.*[92] Detailed information on yeasts and their food value may be found in Chapters 9 and 10 of Volume I, and 6 of this volume.

Synthesis by Candida Yeasts. Burkholder,[5,6,7] in 1943 and later, observed that *Candida guilliermondia* was able to produce significant amounts of riboflavin. In his media, which were largely synthetic, he obtained riboflavin concentrations of up to 75 μg per ml. This fact was likewise observed in 1944 by Schopfer[72] who noted in addition[71] that *C. tropicalis* var Rhaggi was a producer Some irregularities seemed to occur in the riboflavin biosynthetic abilities of *C. guilliermondia*[86] which were shortly explained by Tanner and associates[86,87,92] on the basis of iron control of the biosynthesis. This effect is similar to that of iron on the *Cl. aceto* *utylicum* synthesis[2] of riboflavin. The optimum concentration of ron for the *Cl. acetobutylicum* synthesis is 1 to 3 μg per ml, while or *C. guilliermondia* it was found to be 0.005 to 0.01 μg per ml, hough 0.04 to 0.06 μg per ml was also used successfully.[26] Thus the *Candida* process permits only 1 to 2% of the iron permissible for *Cl. acetobutylicum* processes.

Tanner and coworkers[86,87] found that, with proper control of he iron level of media, two strains of *C. guilliermondia* and one of *C. flareri* were able to produce more than 100 μg per ml riboflavin in 5 to 7 days, *C. flareri* having produced 216 μg per ml. They described a medium containing salts, urea, asparagin, biotin, and glucose. Heavy metals were removed by the 8-hydroxy-quinoline-chloroform method of Waring and Werkman.[94] Iron was then added to the desired level, preferably 0.005 to 0.01 μg per ml and the media were sterilized. The fermentation was conducted with aeration and agitation.

A more practical procedure was also described,[22] in which a 4% glucose solution was passed through a deionizing resin (for example, Nalcite X) for iron removal. Salts, asparagin, and biotin were added, the pH was adjusted to about 5.0, and, after inoculation with *C. guilliermondia,* the medium was incubated for 7 days at 30°C. A riboflavin yield of about 103 μg per ml was obtained in the example cited. The control, which was not resin-treated, yielded 17.5 μg per ml.

In 1949, a modified procedure was described by Levine, Oyaas, Wasserman, Hoogerheide, and Stern[26] who were able to conduct their process without sterilization in plastic vessels. Using *Candida flareri,* they obtained riboflavin yields as high as 567 μg per ml in glucose-containing media, with the average yield being 339 μg per ml. Similar results were obtained when sucrose was used in place of glucose. These authors employed largely synthetic media, such as the one given in Table 17. Satisfactory sterility was generally maintained, since the pH of the medium, which was initially 5 to 6, dropped rapidly to 3.0 to 3.5. This low pH level is antagonistic to most bacterial contamination.

The riboflavin yield paralleled, to a large degree, the yield of yeast. The process was run with aeration and agitation for 4 or 5 days. Dried products were obtained with riboflavin potencies up to 28,000 μg per g for the whole product and up to 97,000 μg per g for the soluble solids.

TABLE 17. MEDIUM FOR RIBOFLAVIN PRODUCTION BY CANDIDA YEASTS

Agent	Quantity
Distilled water, or low-iron tap water	1,000 ml
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Urea	1.84 g
Sucrose or glucose	40.0 g
Biotin[a]	1.0 μg
Zn	140.0 μg
B, Mn, Cu, and Mo	20.0 μg each
Fe, from all ingredients above	40-60 μg

[a] Cane molasses, 0.5 g per l, may be employed as source of biotin if the iron is removed.

Source: Levine, Oyaas, Wasserman, Hoogerheide, and Stern.[26] [Reprinted by permission from *Ind. Eng. Chem.*, **41**, 1665 (1949).]

The extent of commercial interest in *Candida* processes has not been made public. The critical handling requirement with respect to iron is an obvious disadvantage of these processes not inherent in the *Ascomycete* processes.

In 1951, McClary[29a] reported the use of an organism named *Mycocandida riboflavina* which produced riboflavin in glucose corn steep-ammonium sulfate media under acid conditions. Yields of 60 to 200 μg per ml were claimed in an agitated process. The organism is evidently not critical with regard to the iron content of the medium, since corn steep liquor and cane molasses have been employed as raw materials.

SYNTHESIS BY ASCOMYCETES

Outstanding among the riboflavin-producing microorganisms are the two *Ascomycetes, Eremothecium ashbyii* Guilliermond and *Ashbya gossypii* (Ashby and Nowell) Guilliermond *(Nematospora gossypii).* Both organisms are capable of synthesizing very large amounts of riboflavin under appropriate conditions.

In 1947, Moss and Klein[40] reported biosynthesis of riboflavin as high as 2,480 μg per ml by *E. ashbyii* in media containing ground lentils. In 1949, Pridham and Raper[54,56] noted that up to 1,760 μg per ml could be produced by *A. gossypii* under rather rigid fermentation conditions.

Assuming a beer to contain 2,000 μg of riboflavin per ml calculation shows that 10,000 gal of such a beer would contain about 167 lb of riboflavin. At about $60 per pound,[7a] this quantity would be worth about $10,000, if it were all recovered. With cheap raw materials, such a process is obviously profitable.

In addition to the importance of high-assay beers, it is of great ommercial significance that neither organism is particularly adersely affected by moderate levels of iron in the medium, i.e., evels which are excessive for *Candida* and *Clostridium acetoutylicum* processes. For this reason, iron or steel equipment can e used for handling the beers.

YNTHESIS BY *Eremothecium ashbyii*

Eremothecium ashbyii and *Ashbya gossypii* are plant paraites.[12,14] *E. ashbyii* was first found as a cotton parasite at Berber, udan, by R. E. Massey. Confirmation was made by S. F. Ashby. Guilliermond[12,14] described both organisms in 1928 and 1936. E. *ashbyii* is very much like the description of *Eremothecium ymbalariae* Borzi[1,4,13] which was found on an ivy in Italy in 1888, ut which has not been found and identified since.

Many studies, leading to the identification of *E. ashbyii* as a riboflavin producer of some importance, were made by Guilliermond, Raffy, and others,[14,15,36,37,38,57,58,59] starting about 1935. Guilliermond, Fontaine, and Raffy[15] observed in 1935 that yellowish crystals were found at times in the vacuoles of cells of *E. ashbyii.* This yellow substance was identified by Raffy[57] in 1937 as riboflavin. Recently Grob[16] showed spectroscopically that the riboflavin in the culture media exists chiefly in the free form.

Culture. *E. ashbyii* is a heterothallic *Ascomycete,* but only one sexual form has been described as yet. This is in contrast with *A. gossypii* which is homothallic.

Cultural variations of *E. ashbyii* were discussed in some detail by Schopfer and Guilloud[75] in 1945. These consisted mainly of yellow, flavin-producing variants, a poorly sporulating, cream-colored form, which produced relatively little flavin, and white forms, which produced only traces. The white form was described in 1944 by Ritter.[62] The culture may be carried on wort and other nutrient agar media. Schopfer and Guilloud[75] found that the yellow form constantly produced white variants, but the white form was stable and did not yield yellow variants. Desieve[9] observed that the organism gradually lost its flavogenetic ability.

Nutrition. Studies on nutritional requirements of *E. ashbyii* have been made by a number of investigators and not all of the published information is in agreement. Intensive studies were undertaken by Schopfer[70,73] in 1944 and later. Under his conditions,

biotin was reported to be essential for proliferation while *i*-inosi
and thiamin were found to be supplementary factors. These
servations were disputed by Dulaney and Grutter[9a] who fou
an absolute requirement for *i*-inositol, but not for biotin
thiamin. These latter findings were supported by Yaw.[102] Oth
unidentified factors for growth or riboflavin formation by *E. a*
byii were noted in Norite-treated peptone,[70,73] wheat germ,
potato extracts,[70,73] yeast,[23a] rice embryo extracts,[23a] milk pr
ucts,[9,19a,19b,45,46] lipids,[46] gliadin,[19b] and liver extract.[19b]

In their search for satisfactory carbohydrate nutrients for
ashbyii, Schopfer and Guilloud[75] found glucose, sucrose, levulo
and mannose to be useful. Tabenkin,[85] however, employed,
addition to those mentioned, glycerol, mannitol, sorbitol, malto
invert sugar, and certain others. In 1952 Larsen[23b] showed th
in stillage media, the use of a glucose plus a maltose suppleme
resulted in higer riboflavin yields than were obtained with eit
sugar supplement used separately. For example, in a medium e
ploying wheat-flour stillage from the ethyl alcohol fermentati
a 7-day, aerobic *E. ashbyii* fermentation resulted in a ribofla
yield of 418 μg/ml in the control with no sugars added, up
813 μg/ml with glucose supplements of up to 5 g/100 ml, a
1,350 μg/ml with 0.5 g/100 ml of glucose and 2.5 g/100 ml of ma
ose. Lower yields were obtained with maltose alone. The malto
glucose mixture was employed effectively also in casein-contain
and certain other media.[19a,19b]

Studying nitrogen metabolism, Schopfer and Guilloud[75] fou
that arginine, asparagine, ammonium tartrate, ammonium citra
and ammonium succinate were among the useful simple nitrog
sources. The same authors[74] were unable to grow *E. ashbyii* on
medium containing glycine, glucose, mineral salts, biotin, thiam
and *i*-inositol. The addition of L-leucine or L-arginine or both su
ported growth to some extent. About 25 μg riboflavin was form
per ml. Studies were also made, in 1944 and 1945, by Renaud a
Lachaux[61] who obtained 159 μg/ml riboflavin in 24 days, usi
nonsynthetic media.

The cultures of Dulaney and Grutter[9a] did not utilize glyci
as a nitrogenous substrate either, under the conditions describe
but L-(–)-proline, L-(+)-arginine, and DL-glutamic acid allow
growth and low-level riboflavin formation if *i*-inositol was prese
Yaw[102] found histidine to be advantageous in combination wi

ionine. She also studied D-ribose, alloxan, and other potential ursors and found none to be effective.

In 1950, the Japanese investigators Minoura and Kozima pub- ed a series of papers[23a,35a,35b,35c] on nutrition of *E. ashbyii* and he relationship of growth to riboflavin production. Minoura[35a] id that both *i*-inositol and phytin, which occur in rice bran, as growth stimulants. Phytin, however, exerted its effect only nedia containing suitable nitrogen sources. It was presumed phytase formation by *E. ashbyii* is dependent on the utility of nitrogen source in the medium, which, therefore, controls the version of phytin to inositol. Minoura[35b] also sought an active ritional principle in peptone. He observed that since deaminized tone hydrolyzate affects growth only slightly, but does not affect flavin formation, the principle is probably an amino com- ind. Asparagine and arginine were found to affect growth, but riboflavin production. Kozima,[23a] studying extracts of a num- of natural products, found that yeast and rice-embryo extracts e particularly effective growth promoters. Vitamin requirements e also studied,[35c] with vitamins B_1, B_6 and pantothenic acid wing some enhancing action beyond that observed for inositol l biotin. It was concluded that riboflavin formation did not allel the extent of mycelial growth.

Perhaps some of the apparently contradictory findings by dif- ent investigators might be related to the use of different or dified strains of *E. ashbyii* which may have different require- nts. In some instances, conclusions were drawn on the basis of dies in which relatively low levels of riboflavin were produced der the optimum conditions cited. Multiple deficiencies may ve been involved, complicating the problem considerably. The portance of lipids[46] in nutrition has not always been recognized.

Very recently, MacLaren[28a] studied the effects of pyrimidines l purines on riboflavin formation by *E. ashbyii* and suggested cursor action. Xanthine, adenine, guanine, uric acid, and per- ps hypoxanthine promoted riboflavin formation, with xanthine ng the most effective. In contrast, uracil was observed to sig- icantly suppress riboflavin formation. Competitive inhibition s suggested. Yaw[102] found xanthine to be ineffective.

The existence of a heat-labile factor for *E. ashbyii* was noted Desieve.[9] This was found in milk products, such as casein, and s destroyed on extended sterilization. (See also under *A. gos-*

[illegible]). In 1949, Phelps[illegible] found that casein had a unique unu
advantage over other milk ingredients in promoting ri
formation by *E. ashbyii*. Lactalbumin was not as effective
also showed[illegible] that certain lipids, when added to sever
teinaceous media, produced very marked improvements i
flavin yield, *i.e.*, from about 500 μg per ml without the l
about 1,000 or more μg per ml when a lipid, such as corn
butterfat, was included in the medium to the extent of 0.6 t
Rudert[88] had previously observed that lipids could replace
hydrates as energy sources in media, but Phelps'[illegible] work s
growth-factor type of response.

A "synthetic" medium for the cultivation of *E. ashbyii*
allowed formation of over 500 μg riboflavin per ml was de
by Tabenkin[83] in 1950. (See under Commercial Methods
medium contained sucrose, ammonium salts as nitrogen s
and other salts. Nitrogen, in the form of nitrate or nitr
nutritionally ineffective. The sterilized medium was ino
with 4% by volume of a 2-day liquid culture (not detailed
was then aerated and agitated, yielding 571 μg riboflavin
in 5 days. The inclusion of *i*-inositol in the medium rai
yield to 714 μg per ml in 6 days. Essential elements in assi
form, in addition to carbon, phosphorous, sulfur, and amm
nitrogen, were indicated as zinc, potassium, copper, iro
magnesium. Calcium carbonate or certain other agents we
ployed as buffers, the preferred pH range being 5.5 to 6.0.
interest that Tabenkin added no biotin or thiamin as such
media. These factors may have been introduced into the m
by way of the carbohydrates or in the inoculum.

In 1953, studies were described[illegible] which resulted in a
"synthetic" or reproducible medium which was capable o
porting a riboflavin yield of up to about 1,000 μg/ml. Th
thetic" basal medium involved is given in Table 18. This c
of sugars, vitamins, salts, an amino acid, and an oleate and r
in riboflavin yields of up to 500 μg/ml in *E. ashbyii* fermen
Replacement of 1 g of the 20 g/l glutamate with certain o
nitrogenous agents, e.g., casein and gliadin, resulted in a
in riboflavin titer to about 800 μg/ml. Certain other protei
agents were not effective, e.g., peptone, gelatin, and ze
last two agents even suppressed riboflavin formation to a l
80 μg/ml. Not all casein samples, i.e., from different s

ually effective; one of the best studied was a "vitamin-free" Strepogenin did not appear to be involved. Replacement e glutamate of the medium of Table 18 with casein resulted avin yields of up to 1,500 μg/ml. At this casein level, 20 g/l. of casein had relatively little effect on the riboflavin yield arison with differences noted where 1.0 g casein plus 19 g dium glutamate per liter was used.

BLE 18. GLUTAMATE BASAL MEDIUM FOR RIBOFLAVIN PRODUCTION BY *Eremothecium ashbyii*

Agent	Quantity per liter
Monosodium L-glutamate	20 g
Maltose	10 g
Glucose	15 g
K_2HPO_4, KH_2PO_4, and NaCl^b	1 g each
$MgSO_4 \cdot 7H_2O$	0.7 g
$CaCl_2$^{a}	0.5 g
$MnSO_4 \cdot 4H_2O$^{a} and H_3BO_3^{a}	20 mg each
Sodium oleate	0.6 g
i-Inositol	30 mg
Thiamin	1.5 mg
Biotin	0.7 μg
Distilled water	to 1 liter

pH = 6.2

iality not established.

Hickey.[illegible]

as also observed[illegible] that while oleate appeared to act ef- as a growth factor or metabolite for *E. ashbyii* in an e reasonably adequate medium, yeast extract was more than sodium oleate on a weight basis in stimulating ribo- oduction. It was concluded that there were probably at as yet unidentified nutritional factors for *E. ashbyii*, one be- lied by certain complex nitrogenous agents and the other extract, although oleate in appreciable quantity can e for lesser amounts of yeast extract. Further studies lt in an adequate, chemically completely defined medium ll support maximum riboflavin formation by *E. ashbyii*.

mercial Methods. Descriptions of commercial methods vin production are primarily limited to patent literature. not be surprising to find that there are secret methods modifications of described methods.

of the methods to be described here may be in operation haps some of them were operated and have been changed

or modified because of changes in prices of raw mater
other reasons. The methods described can be considere
mercial, potentially commercial, or the bases of opera
mercial processes.

Methods for cultivation of *E. ashbyii* may be surfac
exposed to air, or submerged methods in which air is
throughout the mash, with or without supplementary a

In 1915, Okabe, Hatsuda, and Ueda[44] described
employing a rice-germ medium for surface culture in
1949, in the United States, Martin[89] patented a metho
face culture of *E. ashbyii* on a porous, wet, solid mediu
a wheat-bran or oat-hull medium containing additional
Whole dried residues were obtained containing ribofl
concentration of 3,000 to 4,000 μg per g, along with ot
plex agents. Other related procedures have been re
which soybean-oil cake, other seed cakes, rice and ba
and different brans were used as supporting media.[90]

Most patented methods employ submerged culture
byii in aerated liquid media. A good liquid or submerg
process has the obvious advantages over a solid- or
medium process of efficient use of space and investmen
of transmission of mash or fermented medium ("beer") fr
to another. The following methods will emphasize the f
ashbyii is a truly remarkable and most valuable micro

In 1946, Piersma[47] claimed riboflavin yields up to
ml in liquid media composed of animal proteins, carb
and malt extract. The proteinaceous substances were
glandular materials, tankage, fish meal, etc. Sugar-conta
stances, such as molasses and corn sirup were employed
1%, and malt extract, at 1.5 to 2%. The initial pH v
6.5 and incubation was at 27°C with aeration.

In 1947, Foster,[41] in a British patent, described a
lustrated by the following example. The medium was
of 1% yeast extract, 6% crude dextrose, 0.2% potass
phate, and 0.05% magnesium sulfate at an initial pH
5.6. This was sterilized for 15 minutes at 15 lb steam
cooled, and inoculated with 1% by volume of an aerated
E. ashbyii. The medium was then aerated for 5 days
30°C. At the end of this period, centrifugation
supernatant medium containing 412 μg of riboflavin per

at other B-vitamins, particularly pantothenic and nico-
ls, were also obtained following fermentation.
939, Stiles[34] patented a process for the production of
by the fermentation of several nutrient media with *E.*
The patent discloses the use of stillage ("slops") from
l alcohol and butyl alcohol-acetone fermentations in
ubmerged culture with or without nutritional adjuncts.
ered, in addition to the fermentation residues, are fish
ed yeast, skim milk, cottonseed meal, soybean meal, peas,
and other agents. When these were employed, the enzyme,
" was cited as being used to treat the raw materials prior
ntation. Sterilized media were inoculated with, for ex-
.5% by volume of a culture of *E. ashbyii* and the fermen-
as allowed to proceed in submerged culture with aeration
t 90 hours at about 29°C. Some examples of the Stiles
are given in Table 19. Stiles also mentioned the use of
h protein substances (see Rudert[33]). The highest ribo-
eld shown by Stiles was 436 μg per ml which was produced
pplemented, thin, grain-stillage medium. Moore and de
also noted the use of stillages in 1947; a patent[35] was
1951 describing the use of fortified thin stillage to yield

19. SOME RAW MATERIALS EMPLOYED IN AMPLES OF STILES' PROCESS FOR RIBOFLAVIN PRODUCTION BY *E. ashbyii*

	pH		Riboflavin yield,
	Initial	Final	μg per ml
stillage (TGS), alcoholic	5.5	8.9	290
% high-test molasses	5.4	7.4	352
% corn-steep liquor + 0.2%			
added at 42 and 78 hours	5.8	7.5	436
at scraps + 0.75% glucose	6.5	7.6	128
red wheat	6.5	7.6	158
tage + 0.75% glucose	6.5	7.6	162
d field beans	5.5	8.1	178
gluten meal	6.5	7.1	141
	6.5	7.8	180
ean meal	6.5	7.8	130
seed meal	6.5	7.6	105
meal + 0.75% glucose	6.5	7.9	225
t middlings	6.5	7.6	214
ed-oil meal	6.5	8.0	118
d yeast	6.5	7.6	207
(hand skimmed)	7.0	7.5	406
d skim milk (liquid)	7.0	7.5	250

tiles.[34]

a product containing 2000 to 10,000 μg of riboflavin per of solids. A process employing whole peanut flour, supple with a carbohydrate, was described recently by Beesch and T It is of interest that stillages were also employed as substra B-vitamin synthesis by *Aerobacter* species.[illegible]

Another process involving the use of cereal grain was pa in 1950 by James.[illegible] In this procedure, a highly malted mash was employed a a substrate for the aerobic cultivat *E. ashbyii*. Riboflavin yields in excess of 1,000 μg per ml obtained from media containing about 12% initial grain so

In 1948, a British patent issued to Chas. Pfizer & Co., Inc scribed one of the higher yields reported for *E. ashbyii*. A m cited contained 5% brown sugar, 3% neo-peptone (Difc wheat germ, 0.5% beef extract (Difco), 0.5% KH_2PO_4 and NaCl made up in distilled water. The initial pH was 6, the lum 10%, the temperature 28°C, the fermentation period 7 and the yield 1,800 μg per ml. Agitation was employed in ad to aeration.

More recently, Larsen[illegible] demonstrated that in the ferm tion of stillages from the alcoholic fermentation of cereal g the use of sugar mixtures as more advantageous than that of sugars. The preferred mixtures contained maltose supplem with glucose, sucrose, or levulose. In an example, whea stillage, supplemented with 0.5 g glucose and 4 g malto 100 ml of medium, yielded 1,610 μg per ml riboflavin after 1 of submerged fermentation with *E. ashbyii*.

In 1949, Phelps obtained two patents[illegible] which are o siderable interest. One of these[illegible] describes media con casein or other milk products, commercial malt extract, an cose, which, on fermentation with *E. ashbyii* in subm aerated culture, yielded in some cases over 600 μg riboflav ml. In one example, a basal medium containing 1.75% com malt extract and 0.5% cerelose (glucose hydrate) was supple with several milk products which contained casein. After 88 at 30° to 32°C, the riboflavin yields were 310 to 690 μg per ml

Phelps[illegible] demonstrated that casein was the primary cons of milk which was responsible for high riboflavin yield showed that the malt extract-cerelose medium (see before), supplemented with skim milk, casein, whey plus casein, plus lactalbumin, or whey plus casein plus lactalbumin

nation, yielded riboflavin in the range of 532 to 612 μg per
/hen the supplements were whey or lactalbumin, the yields
92 to 232 μg per ml.

his other patent, Phelps[46] supplemented his skim milk,
xtract, glucose type of medium with butterfat and showed
hen about 1% butter fat was added to the medium, the
vin yield amounted to 1,120 μg per ml in 88 hours, compared
control containing no butterfat which yielded 455 μg per ml.
also studied the effect of other lipids. These were added
asal medium which contained 1.75% (dry basis) malt ex-
0.5% glucose, and 1% casein solubilized by treatment with
nia. (A 10% aqueous suspension of casein was heated for
at 45° to 50°C in the presence of 0.7 ml of 26% ammonia
of casein.) The lipids studied included corn oil, soybean
oconut oil, lard oil, oleomargarine, lecithin, menhaden
d others. They were added to the medium at a concentra-
of 0.6 to 1.25%. Riboflavin yields ranged from 810 to
g per ml, as compared with a control having no lipid sup-
nt which yielded 420 μg riboflavin per ml.

1952, Hickey[19a] described a patented process which em-
sodium oleate, along with other nutrients, in casein media
sulted in riboflavin yields of up to 1,370 μg per ml after 5
submerged, aerated cultivation of *E. ashbyii*. Both maltose
ucose were employed as carbohydrates.

elps[46] also described the effect of lipids on proteinaceous
other than casein or lacteal media. Table 20 shows the
on the riboflavin yield of supplementing several types of
naceous media with several lipids. It is evident from the
Table 20 that the addition of lipids to various media will
te riboflavin synthesis by *E. ashbyii* to the extent of a
increase or more in some instances.

1950, Tabenkin[85] described a "synthetic" medium for
vin production by *E. ashbyii*. His basal medium contains,
mple, per liter of medium: 50 g sucrose, 3 g $(NH_4)_2HPO_4$,
CO_3, 2 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g $FeSO_4 \cdot 7H_2O$,
$ZnSO_4 \cdot 7H_2O$, 0.005 g $CuSO_4 \cdot 5H_2O$. A pH range of 5.5 to
vas preferred. This medium, after sterilizing, was inoculated
1% by volume of a 2-day liquid inoculum of *E. ashbyii*. The
n, on vigorous aeration and agitation at 28° to 30°C for 5
elded 571 μg of riboflavin per ml. The same operation, but

with a supplement of 0.05 g of inositol per l yielded 714 µg pe in 6 days. It is of interest that Tabenkin used essentially no plex nitrogen in the medium.

A process for riboflavin synthesis by *E. ashbyii* in exce 2,000 µg per ml was covered by a British patent in 1947. This the procedure of Moss and Klein[illegible] of Roche Products Ltd., found that good yields of riboflavin could be produced by *E. byii* in submerged culture and media containing lentils *esculenta*) and a fermentable carbohydrate. Control of pH at a 7 by intermittent addition of cane molasses, and the use of monium salts of certain organic acids favorably influenced re Incubation was at 28° to 30°.

TABLE 20. ENHANCEMENT OF RIBOFLAVIN YIELD *Eremothecium ashbyii* BY INCLUSION OF LIPIDS

Medium	88-hour riboflavin yield, µg per			
	No lipid added	Cream added	Corn oil added	Pear ad
Basal[a] + 8% fish meal	818	1,295	1,122	1
Basal[a] + 2% peptone	290	575	440	4
Basal[a] + 5% distillers' solubles	560	985	1,040	9
Basal[a] + 8% liver cake[b]	410	880	815	1
Basal[a] + 1% solubilized casein[c]	580	960	1,015	9

[a] Basal contained (dry basis) 1.75% malt extract and 0.5% glucose, all adjusted at pH 6.5 to 7.0 before sterilizing.

[b] Digested at pH 9.5 with a proteolytic enzyme.

[c] Solubilized by ammonium hydroxide.

Source: Phelps.[46]

Examples of the method of Moss and Klein are presente Table 21. Inoculum was 10% by volume of a heavy mycelial pension. Octadecanol in paraffin oil was employed as an anti

It is possible that riboflavin yields by *E. ashbyii* may ex 2,500 µg per ml. The high yields reported are quite ama They are also unique in the field of microbiological vit synthesis.

SYNTHESIS BY *Ashbya gossypii*

Ashbya gossypii (Ashby and Nowell) Guilliermond, called *Nematospora gossypii*, is a parasite of some importan cotton, coffee, and certain other plants.[illegible] In 1928, liermond[11] had observed the similarities of *E. ashbyii* and *A. sypii*. In 1935, Guilliermond, Fontaine, and Raffy[illegible] had found riboflavin was produced rather weakly by *A. gossypii*. This was

TABLE 21. RIBOFLAVIN PRODUCTION BY *Eremothecium ashbyii*

n	Fermentation, days	Temperature, °C	Riboflavin found, µg per ml
ground lentils, 1% cane mo- s, 0.5% NaCl[a]	8	28	1,400
ground lentils, 5% cane mo- s, 0.5% NaCl[b]	7	28	1,100
ground lentils, 0.75% D.B.[c] ose, 0.25% inverted D.B. ose, 0.3% ammonium succinate, 0.5% NaCl	8	28	1,700
ground lentils, 0.75% D.B. ose, 0.25% inverted D.B. su- e, 0.27% ammonium succinate, % sodium succinate, and 0.5% l	7	28	1,810
ground lentils, 1.125% D.B. ose, 0.375% inverted D.B.[d] su- e, 0.5% ammonium succinate, 0.5% NaCl	5.7	28	2,480

dium was autoclaved for 30 minutes at 120°C and 2 days later again.
tils had been steamed for 3 hours prior to use.
k brown.
ne molasses or brown sugar solutions were added from time to time as of maintaining the pH between about 6.8 and 7.5. Without this supple- on, the pH rose above 7.5.
e: Moss and Klein.[60]

nding of Schopfer and Guilloud.[76] In 1946, Wickerham, nger, and Johnston[93] reported that a strain of *Ashbya ii* (NRRL Y-1056), which they obtained in 1943 from W. bins, yielded a variant whose colonies on agar produced a orange-yellow color after a few days at room temperature. 0, Pridham and Raper[58] published a review on *A. gossypii* covered occurrence, pathogenicity, taxonomy, cultural, ological, and physiological characteristics, along with brief ation on riboflavin production.

ickerham, Flickinger, and Johnston[93] pointed out that the yellow variant[58] produced riboflavin to a significant ex- the presence of moderate amounts of iron in contrast *andida* yeasts and *Clostridia* which required very low iron o form important amounts of riboflavin. They showed that nedium composed of 0.3% powdered-yeast extract, 0.5% e, and 2% Cerelose (glucose) at pH 6.8 to 7.0 in aerated ory fermentations, yields up to about 380 µg per ml were d in 8 days. The addition of 0.3% distillers' solubles and

0.1% $CaCO_3$ improved results somewhat. They also repor
a different lot of yeast extract gave poor riboflavin yields,
because of deficiency in certain factors.

In 1947, Tanner and Van Lanen[illegible] noted briefly pr
conditions for riboflavin formation by *A. gossypii*. The
procedure was patented in 1948 by Tanner, Wickerham, a
Lanen.[illegible] Tanner, Vojnovich and Van Lanen[illegible] detaile
findings more completely in 1949, with laboratory and pil
scale studies made at the Northern Regional Research Labo

The optimum temperature range for riboflavin pro
was found to be between 26° and 28°C. Although the o
grew well over a fairly broad temperature range, it was
that at higher temperatures, the yield was reduced, while a
temperatures, excessive fermentation periods were require

An initial pH of 6.0 to 7.0 was found to be prefera
starting ph of 4.5 to 5.5 allowed a good growth, but the ri
yield was less satisfactory. At an initial pH of 4.0, both grow
riboflavin formation were quite poor.

A. gossypii was found to be rather discriminating re
satisfactory carbohydrate substrates. Glucose was quite satis
Fairly pure forms of sucrose and maltose were also en
satisfactorily, while crude agents, such as cane or beet mol
hydrol, were unsatisfactory because of poor riboflavin yie
though mycelial growth was quite heavy. Pentoses did not
growth well, nor did starch or modified starches. Dur
fermentation, glucose is largely consumed before riboflav
to appear in quantity.[illegible] Other carbohydrates have als
studied.[illegible] Carbohydrate metabolism of *A. gossypii* was
in 1950 by Mickelson.[illegible]

Regarding media, Farries and Bell[illegible] reported nutriti
formation in 1930. In the following years, studies were m
a number of other investigators. These were reviewed in
Pridham and Raper.[illegible] Essential for the growth of *A. gos*
i-inositol, thiamin, and biotin. Robbins and Schmidt[illegible]
that *A. gossypii* grew rapidly in a synthetic-type medium c
of glucose, asparagin, biotin, thiamin, i-inositol, and sever
A significant amount of riboflavin did not appear to form
is evidently not assimilated, and the ammonium ion appears
little nutritional value.[illegible] More complex media,[illegible] containi
tone, yeast extract, and sugar, resulted in much better ri

. Lipids may be of importance for high riboflavin pro
A. gossypii. The use of lipids was indicated, but de
not supplied. Lipids were shown by Phelps to be of
le importance in an Eremothecium ashbyii process. E.
s also reported to give good riboflavin yields in media
he nitrogen was supplied by ammonium salts.

er, Vojnovich and Van Lanen of the Northern Regional
Laboratory showed, in 1949, that corn steep liquor was a
y and economic agent which could replace yeast extract
edia. Distillers' solubles could also be used. As sub-
r peptone, vegetable proteinaceous agents, such as soy-
, glutens, linseed, and cottonseed meals, were investigated
found to give results which were erratic and inferior
obtained in a corn steep-peptone type medium. Pro-
s agents from animal sources were found to operate
ctively in corn steep-glucose media. Among the useful
re tankage, animal stick liquor and meat scraps. Acid-
e-hydrolyzed proteinaceous agents gave considerably bet-
than the unhydrolyzed product. Fish stick liquor
tisfactory because of very low yields of riboflavin. As a
medium containing 4% glucose, 0.5% corn steep liquor,
peptone yielded 395 μg of riboflavin per ml in 8 days.
ef tankage was substituted for peptone, a 7-day yield of
r ml was reported. It was mentioned that by the generally
d procedure, with some modifications which were not
yields in excess of 1,000 μg per ml were obtained. The
day reported by these investigators was 1,420 μg per ml
s obtained in a 30-l fermentation. Very recently, Bersch
an reported a process using unhydrolyzed plant proteins
lded about 1,000 μg/ml.

g with the importance of nutrition of A. gossypii for ob-
od riboflavin yields, Tanner, Vojnovich, and Van Lanen
ut in 1949 that excessive sterilization of media reduced
e eventual riboflavin yield. Data showing this are pre-
Table 22. Flash-sterilization or continuous-cooking
s would, no doubt, be important here; sterilization of
s separately was one means of avoiding the effect. Brief
n on a large scale is probably best handled by some
er than batch cooking.

ame authors also found in inoculum development studies

TABLE 22. EFFECT OF DIFFERENT STERILI
PERIODS ON RIBOFLAVIN YIELD BY *Ashbya g*

Autoclave period at 121°C, minutes	Fermentation age	
	8 days, µg per ml	10 da µg per
0 (Seitz filtration)	678	676
15	648	700
30	680	700
45	494	588
60	308	340
75	288	300
90	248	324

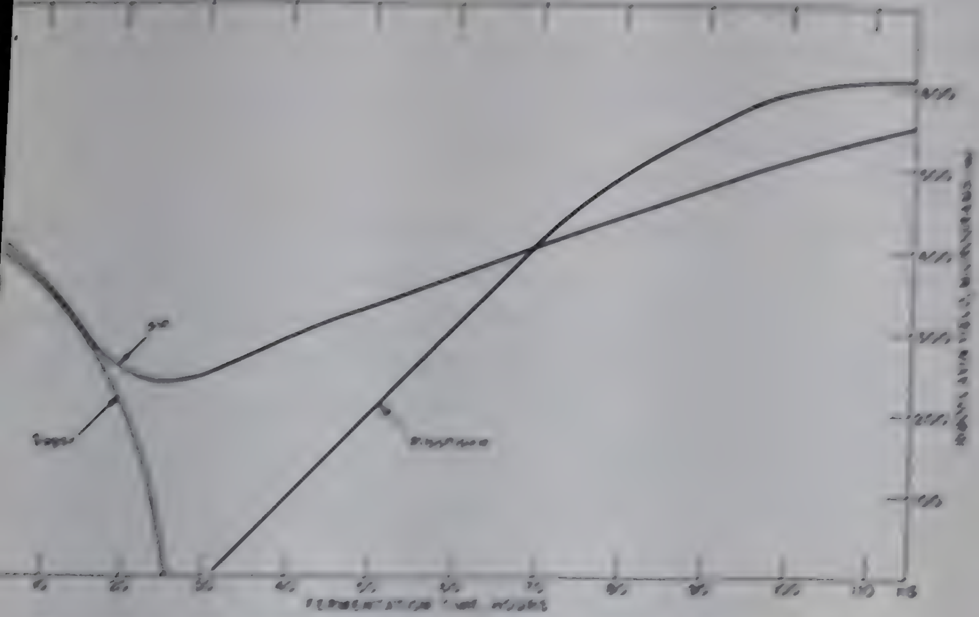
Source: Tanner, Vojnovich and Van Lanen[illegible]

that the age and proportion of inoculum influenced the
yield considerably. A 24-hour inoculum was found to
preferable to 72-, 96-, or 120-hour inocula. The preferre
of inoculum was 0.5 to 1.0% by volume. Higher levels
satisfactory and a 10% by volume inoculum resulted i
lower yield. Efficient aeration was essential.

Pridham and Raper[illegible] and Pridham[illegible] pointe
1949, 1950, and 1951 that with very critical control o
and with a higher nutrient level, including corn-ste
yields of 1,200 µg per ml were obtained from *A. goss*
consistently. With glucose feeding, a peak of 1,760 µg p
obtained. An inoculum of 0.25% by volume was u
found no superior strains through strain selection.

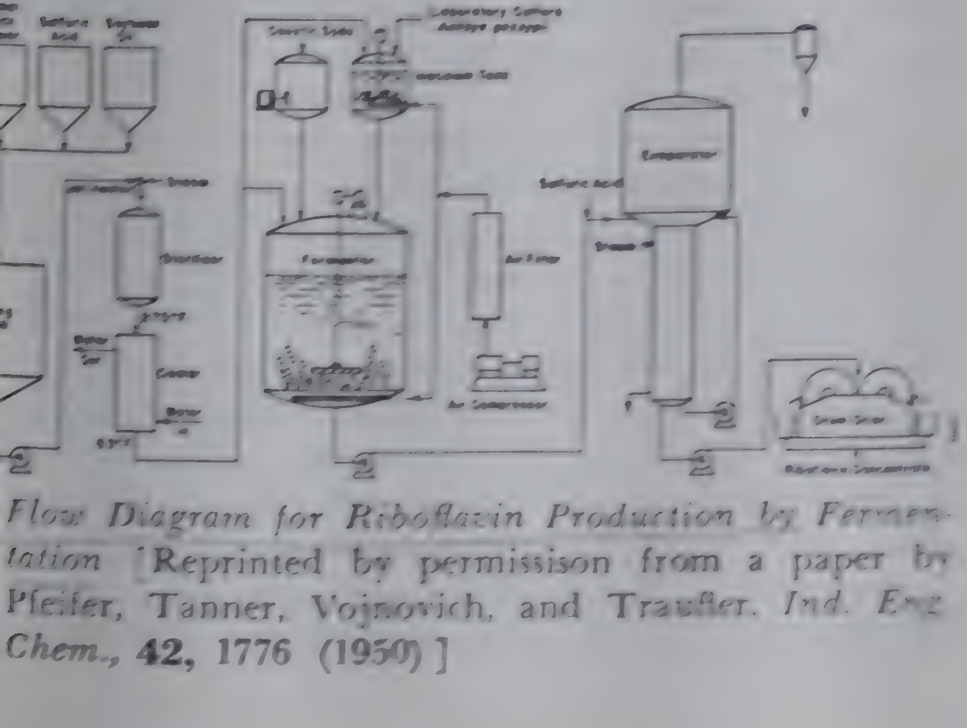
In 1952 Pridham and Raper[illegible] described studies on
and mutation of *A. gossypii*. They noted a general te
the organism to form degenerate types which are less e
riboflavin producers. The inclusion of 0.1% sodium di
stock-culture media enhanced pigment formation.

Pfeifer, Tanner, Vojnovich, and Traufler[illegible] des
A. gossypii process on a pilot-plant basis in 1949 and 19
were also presented in a 1950 pamphlet of the Norther
Research Laboratory by Pridham, Hall, and Pfeifer.[illegible]
tations were operated satisfactorily in glass, aluminu
and stainless-steel equipment. A medium was used w
tained 2% glucose, about 2% corn-steep liquor, and 1
stick liquor at pH 4.5. As an antifoam, 0.1 to 0.2% s
was included. This was sterilized for 5 minutes at 275
continuous procedure. The medium was inoculated w



Changes in Medium during Fermentation [Reprinted by permission from a paper by Pfeifer, Tanner, Vojnovich, and Traufler, *Ind. Eng. Chem.*, **42**, 1776 (1950)]

by volume of seed culture. The medium was aerated agitated for 4 or 5 days at 28° to 29°C. The riboflavin 500 to 600 μg per ml. The resulting product was con-and dried on a drum drier, yielding a 2.5% riboflavin timated to cost 3.75¢ per gram of riboflavin. Dried fer-



Flow Diagram for Riboflavin Production by Fermentation [Reprinted by permissison from a paper by Pfeifer, Tanner, Vojnovich, and Traufler. *Ind. Eng. Chem.*, **42**, 1776 (1950)]

mentation products having a riboflavin potency of
50,000 μg per g are reported.[58] Crystalline riboflavin c
tained. Figure 6 shows characteristic changes in the me
ing fermentation and Figure 7 is a flow sheet of th
process.[48a]

Recently, Smiley and coworkers[75] found that *A. gos*
duced vitamin B_{12} or a B_{12}-like agent; also the *La*
bulgaricus factor[75,88] is formed in quite appreciable am
Although *Eremothecium ashbyii* and *A. gossypii* ar
logically similar[24] and both evidently require inositol, b
thiamin,[25,65,70,92] the general types of media used for hig
flavin yields from the two organisms are considerably d

RECOVERY OF RIBOFLAVIN FROM FERMENTED PRODUCTS

Fermentation liquors containing riboflavin may be
for use as animal- or human-food supplements simply
and powdering. This is true of butyl alcohol-acetone fer
residues, among others. Drying is generally accomplish
practicable by drum- or spray-drying procedures. These
tions usually contain other members of the B-comple
with minerals and proteinaceous substances.

Some liquors, or "beers," may contain sufficiently
centrations of riboflavin to make isolation of pure
riboflavin profitable. This is particularly true of liq
the *E. ashbyii* and *A. gossypii* processes which may ex
μg per ml in potency.

Two methods were described in 1945 by Hines,[90,91] w
first that by bacteriological reduction, up to 90% of the
in *E. ashbyii* fermentation liquors could be precipitated
dish-brown, amorphous product of 80 to 90% purity.[18]
rus faecalis, S. liquefaciens, and certain other bacteria
The method was described further in 1946 by Hickey,[77]
cussed the nature of the precipitate. Hines then foun
similar result could be obtained more rapidly, almost in
in fact, by the use of chemical reducing agents, such
diphosphite ($Na_2S_2O_4$), stannous and chromous chlorides
tain other reducing compounds. A modification of the
precipitation-recovery system was reported by McMill

ed the riboflavin in the presence of a filter aid, such as
. This allowed a very easy and rapid recovery of the
e by press filtration and also assisted settling where this
able. Of the chemical reducing agents, sodium dithionite
lfite) has economic and low toxicity advantages over some
other operative reducing agents, such as stannous or
chlorides.

crude precipitate which is obtained by such means can be
onverted to crystalline riboflavin, for example, by the
f Dale.[8] In this process, the precipitate is dissolved in
ar solvent, such as 50% aqueous isopropyl alcohol. The
s filtered and yields a greenish filtrate, containing ribo-
a reduced state. The reduced riboflavin is oxidized, by
xample, and the oxidized riboflavin precipitates as yellow

re have been many methods for recovering riboflavin
ous sources. Some of these have been reviewed by Rosen-
Many of the methods were applied to raw materials having
w potency compared with preparations from *E. ashbyii* or
bii which may contain 1,000 to 2,000 μg or more per ml.
chemical precipitation methods of Hines[26] and McMil-
rate primarily on solutions containing much riboflavin;
20 μg per ml. *E. ashbyii* beer filtrates operate well, but
boflavin solutions can also be precipitated.[12]
ecent method for the recovery of riboflavin from low-
preparations has been described by Keresztesy and Rickes.[22]
loyed butyl alcohol for extraction of riboflavin directly
e fermentation substance. Repeated extractions were
.

949, Legg and Cranmer[23] described a method for ap-
to high- or low-concentration (i.e., less than 20 μg per
parations. The effective operation of this method de-
the fact that reduced or leuco riboflavin is much easier
t from aqueous solution by certain alcohols, e.g., *n*-butyl
han is the oxidized form. Alcohols which can be used are
tially miscible with water. Isobutyl, secondary butyl,
benzyl, and cyclohexyl alcohols are among those cited,
tyl alcohol is preferred. An example of the method is as
A stillage containing about 10.6 μg of riboflavin per ml
t 0.91% of other solids was reduced with about 0.02%

sodium dithionite (hydrosulfite), and was mixed with volume of *n*-butyl alcohol in an atmosphere of carbon After shaking and standing, the phases separated. Assay ing reoxidation showed about 76% of the riboflavin in the phase. The potency on a dry basis was 10,700 μg per g with 1,164 μg per g in the original substance. When the was run without reduction, only 37% of the riboflavin in the *n*-butyl alcohol phase. The solids in this extract 5,500 μg per g, or about half the potency of the dry pared by the reduction-extraction procedure. Reducing other than dithionite may also be used.

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CHAPTER 6

PRODUCTION OF VITAMINS OTHER THAN RIBOFLAVIN

J. M. Van Lanen

INTRODUCTION

In recent years, remarkable advances have been made in our knowledge of the vitamins—their distribution in nature, chemistry, biological functions, and biosynthesis. Microorganisms, as tools of study, have come to play a prominent part in gathering this knowledge and applying it to practical nutrition problems. They have been especially useful in the discovery and elucidation of various members of the B-complex family, since these vitamins are not only common to all living cells, but they perform essentially the same biochemical roles in each, i.e., they serve as functional constitutents of enzyme systems which provide the cell with energy and the required structural units. The selection and utility of microorganisms in pursuing nutritional problems is based on this fundamental similarity in cellular metabolism, coupled with the ease and rapidity with which they can be cultivated on substrates of known chemical composition.

For the determination of vitamins and the exploration of specific biochemical reactions, necessarily widespread use has been made of fastidious organisms which are highly dependent on the external environment for their growth requirements. This has served to focus attention on the vitamin deficiencies of fungi. However, within the past decade, it has been demonstrated, both by surveying numerous species for their vitamin requirements and by the actual assay of microbial products, that the great majority of microorganisms is capable of appreciable vitamin synthesis. Those organisms with exceptional biosynthetic ability are receiving ever greater attention both in industry and in the research laboratory.

Vitamins formed during the growth of microorganisms are retained within the cells, degraded during metabolism, or excreted into the surrounding medium in the same manner as other metabolites. As a consequence, in certain environments, both natural and artificial, which support abundant microbial development, vitamins often are demonstrable in substantial amounts. In nature, such loci are waters of high organic content, sewage, soils, especially in the proximity of higher plant life, and the intestinal tracts of animals. The extent to which vitamins so formed contribute to the nutrition of animals and conceivably to the early development of some higher plants is not fully known; however, it is true that all the B-vitamin needs of ruminants can be satisfied once the rumen flora becomes established.

On artificial substrates, i.e., pure-culture propagation, vitamin synthesis occurs together with growth or fermentative activity. Where large volumes of media are processed, as in industrial fermentations, this synthesis substantially enhances the nutritional value of either the fermentation products or the by-products. In recent years, increasing attention has been given to the full recovery and proper utilization of these values. In this chapter, those microbial processes will be discussed in which the vitamins synthesized have commercial or potential commercial importance. For consideration here, these have been grouped as follows:

(1) Biosynthetic processes which result in the vitamin enrichment of the fermentation product, such as yeasts grown for food, pharmaceutical, or feed purposes.

(2) Biosynthetic processes accompanying other fermentations in which the vitamins occur in and add value to the fermentation residues. Several industrial fermentations fall into this

group, the vitamins resulting in vitamin-rich by-products whose recovery has often come to bear importantly on the over-all economics of the process involved. An outgrowth of the recognition of vitamin synthesis in certain fermentations, for example, the acetone-butanol process, has led to a more intensive study of the biosynthesis of vitamins and to improved methods for the production of riboflavin (see Chapter 5).

(3) The use of microorganisms to demonstrate the existence of previously unidentified vitamins and to carry out their synthesis in primary fermentation processes, i.e., those in which the vitamin is the principal product of the fermentation.

HISTORICAL

Microorganisms have been associated with the nutritional well-being of animals dating back to a period which coincides with the origin of the science of nutrition. Yeast was fed to livestock with favorable results during the latter part of the last century and in 1900, reports began to appear concerning its nutritional merits.[47] The work of Hopkins,[26] in 1906, supplied partial confirmation for these claims, since he showed that milk and yeast contained "accessory factors" for rats placed on purified rations. The first specific nutritional entity to be found a component of any microorganism was the antineuritic principle which, in 1910, Schaumann[54] demonstrated to be present in yeast. A few years later, while searching for the antineuritic principle, Funk[16] isolated nicotinic acid from yeast. Although nicotinic acid was inactive against polyneuritis, its isolation undoubtedly attracted further attention to yeast as a source of vitamins. From these early observations until the present time, yeast has continued to rank among the best natural carriers of B vitamins and in several cases (folic acid, *p*-aminobenzoic acid, coenzyme A, etc.), it has been used as a source material for isolation of specific factors. Several reviews have dealt with the vitamin content and general nutritional value of various types of yeast.[45,47,76]

The conviction that other microorganisms synthesize vitamins likewise had an early origin. In 1911, Osborne and Mendel[41] noted that a diet inadequate for rats permitted growth when access was had to the feces of animals maintained on a complete

diet. They attributed this action to the synthetic activitie
microbial flora engendered by the complete diet. In 1914, Coo
showed that alcoholic extracts of hen and rabbit excreta posse
antineuritic activity which led him to postulate synthesis b
testinal flora. Further evidence along this line, and the first
ruminants, was the report of Theiler, Green, and Viljoen
1915 that the rumen contents of cattle contained antine
principle although the diet lacked this substance. They lik
implicated rumen microflora in the acquisition of the vitami

Reports, in 1927, by Fridericia, Freudenthal, Gudjonn
Johansen, and Schoubye[15] and by Roscoe[50] demonstrated th
diets rich in starch, some rats became completely indepe
of a dietary B-vitamin supply. This relationship between die
microbial activity in the intestinal tract has been amply conf
in recent years.[36] The complete independence of rumina
dietary B vitamins was shown by Bechdel, Honeywell, Du
and Knutsen[4] who found that calves could be reared to mat
and were capable of normal reproduction on diets which
deficient in B vitamins for laboratory animals. The impor
of both intestinal and rumen microflora in satisfying the
tional requirements of animals has been discussed from biol
and biochemical viewpoints in recent articles.[24,28,31a,36,66a]

That specific bacteria are capable of synthesizing vit
was first suggested by the finding of Pacini and Russel,[42] in
that culture media, in which typhoid organisms had been
tivated, stimulated the growth of rats. In 1923, Damon[18]
that vitamin B was synthesized by several mycobacteria.
production of vitamin B by *Bacillus vulgatus* also, was rep
by Scheunert and Schieblich[55] in 1927. Beginning about
an increasing number of microorganisms was shown to be ca
of producing definite members of the vitamin B complex. D
the past two decades, attention has been directed both t
quantitative aspect of synthesis by organisms in industria
and to the mechanisms and intermediates of vitamin form
by fungi, in general. These studies which have been summ
by Knight,[31] Peterson and Peterson,[45] and Van Lanen and
ner,[75] provide much evidence to support our present conce
garding the wide distribution and importance of B vitam
metabolism.

NTHESIS OF B VITAMINS DURING INDUSTRIAL FERMENTATIONS

Yeast Fermentations

easts are the principal fermentation product in bakers', r, and food yeast production and a by-product of brewing ertain distillery operations. The details of their cultivation ese fermentations are described in Chapters 6, 9 and 10 of ne I. Whether fermentation main-product or by-product, main use is in the formulation of foods, pharmaceuticals, eedstuffs and thus vitamin content and uniformity are im-nt factors in yeast propagation.

he vitamin composition and the range of potencies of the only grown yeasts is shown in Table 23. It is obvious from ble that yeasts are a comparatively rich source of B vitamins. vitamin B_{12}, but that there are appreciable variations both and between species. While these differences are as yet fectly understood, they can be accounted for largely by one re of the following variables: (a) medium composition, (b) gating conditions, and (c) species or strain used. The man-d extent to which each of these influences vitamin potencies lined briefly as follows.

ABLE 23. VITAMIN B COMPLEX CONTENT OF COMMERCIAL YEASTS*

	Bakers' compressed	Bakers' active dry	Primary grown	Brewers'	*Torula utilis*
ne	16-40	12-29	120-200	60-180	5-53
vin	25-80	31-70	40-80	21-80	25-70
enic acid	150-330	100-150	112-150	42-200	40-180
	240-700	180-300	250-700	281-1,000	153-600
ine	25-65	—	32-40	23-100	35
id	19-80	—	—	19-30	4-31
	0.8-0.24	0.4-1.5	2.5	0.8-1.1	0.5-3.6
obenzoic	24-175	15	10-16	9-102	17-62
	4,320	—	4,000	2,700-5,000	2,700-3,600
	2,100	—	4,000	2,500-5,000	—

* µg per g of dry cells.

M COMPOSITION

has been well established by the use of synthetic media and ubstrates of known vitamin content that yeasts are capable

of the total synthesis of B vitamins. However, higher vi
potencies are usually obtained when media are supplemented
natural products, such as grain extracts, malt sprouts, mo
etc. Such supplements have both direct and indirect effec
vitamin formation. They supply inorganic elements and gr
promoting substances which indirectly promote vitamin syn
Directly and, more important, quantitatively, they provide
vitamins and vitamin precursors which the cells absorb or
vert to vitamins. Compared with other fungi, yeasts are ca
of both absorption and synthesis from precursors to a high d
and each of these properties has been utilized industrially t
pare enriched products of predetermined vitamin content.

ABSORPTION

Thiamine, niacin, and biotin are avidly absorbed by v
pyridoxine and inositol are taken up to a lesser extent, while
thenic acid and some other B vitamins may be absorbed
limited physiological conditions. In unfortified media, a
tion is an important contributory factor to high vitami
tencies only under anaerobic conditions where the yeast
is low in relation to the available vitamins. The brewing
ess is a good example of fortification under anaerobic cond
and the higher levels of thiamine and niacin in brewers'
(see Table 23 and Norris[89]) can be explained on this bas
fortified media, however, propagation may be either aero
anaerobic and enrichment with thiamine can be brought
merely by aerating a cell suspension in the presence of the vi

In one of the patented methods of enriching yeast with
mine,[80] rice polishings, malt sprouts, or other natural vi
containing materials are extracted and the extracts are com
Carbohydrate is added to yield the yeast crop and vitamin p
desired and the medium is fermented in the usual m
Another patented process[60] involves cultivation of yeast in
fortified with crystalline thiamine. The thiamine potency
increased in this way from the normal level up to 600 to
per g with a high efficiency and cells containing over 6,000
g have been produced with a low efficiency. Absorbed thi
especially under anaerobic conditions, is converted larg
cocarboxylase.

Yeasts also absorb niacin[73] and a four- to fivefold
ment can generally be obtained by adding this vitamin

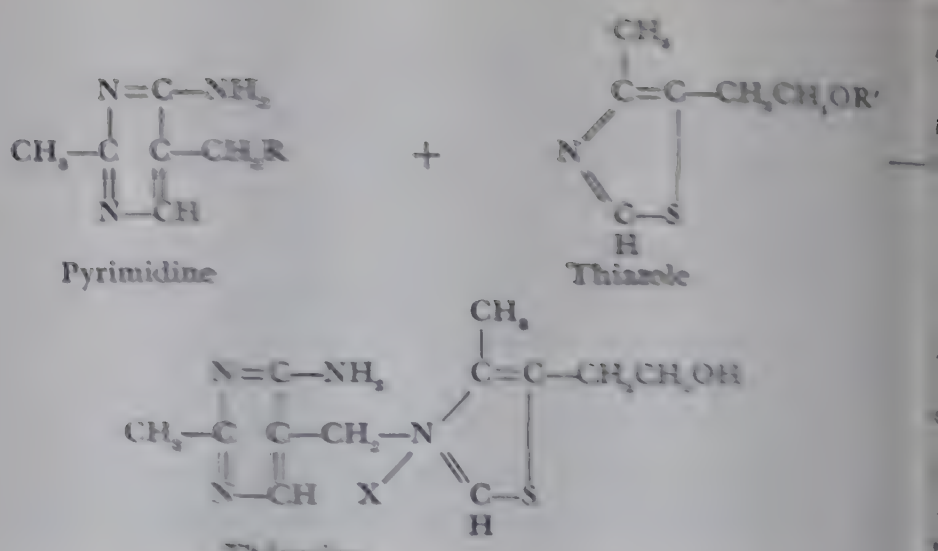
ium. Potencies of 3,000 μg per g have been secured by adding rge excess of niacin. The uptake of niacin, in contrast to mine, requires fermentable carbohydrate and, under anaerobic litions, absorbed niacin is rapidly released following carbo- rate utilization.

Biotin absorption is appreciable with some yeasts, but this erty varies considerably between species and strains.[77] One haromyces cerevisiae culture was increased in biotin content 0.5 to over 200 μg per g by fortifying the medium. Although in, niacin, and thiamine absorption takes place in both syn- ic and natural media, the process is more complete in the nd due apparently to the fact that they supply factors which r absorption.

Riboflavin values are fairly uniform for the yeasts of com- ce, since any excess of this vitamin is released into the medium er than retained following synthesis. A similar behavior is bited in the case of *p*-aminobenzoic acid which is synthesized excreted into the medium.[22] Certain yeasts, i.e., *Hansenula nala* and *Mycotorula lipolytica*,[44] have been found to produce uch as 1 mg of *p*-aminobenzoic acid per gram of cells. Yeasts ommerce are very low in vitamin B_{12} content, ranging from to 0.2 mμg per gram.

THESIS FROM PRECURSORS

Microorganisms are frequently limited in the synthesis of a min because of inability to fabricate a particular portion of molecule. Once this limiting portion or precursor is supplied, synthesis is completed, sometimes in substantial amounts. The indication of biosynthesis from precursors was noted in ies on thiamine production in 1937.[43] It was found that mine, after heat inactivation, was regenerated when added to ures of growing yeast. Knight,[30] about the same time, reported pyrimidine and thiazole could be substituted for thiamine as a th factor for *Staphylococcus aureus*. In 1938, Schultz, Atkin. Frey[58] found that these compounds replaced thiamine for s which were incapable of synthesizing their growth require- ts. These observations culminated in the development, patent- and commercial use of a number of quite similar processes nriching yeasts with thiamine.[22,25,59] The synthesis of thiamine the intermediates involves formation of a methylene bridge een the pyrimidine and thiazole moieties:



Pyrimidine

Thiazole

Thiamine

Compared with the filamentous fungi, yeasts are relat
exacting as to the pyrimidines and thiazoles which they can
vert to thiamine. Only substitutions in the positions mark
and R′ in the preceding formulas are permissible. R ma
an aminomethyl, ethoxymethyl, oxymethyl, hydroxymethyl, a
ethyl, thioformyl, or cyano group.[3,14,25,55] R′ may be hydr
an acyl group, or another group hydrolyzable to yield the hyd
group.

To carry out the biosynthesis, the pyrimidine and thiazol
added to the wort during propagation of the yeast, prefe
during the later stages of growth. By this method, cells ca
enriched from the normal thiamine content of 20 to 40 μg
up to 1,200 μg per g. However, commercial products comn
have been standardized at approximately 630 μg per g. At
level of enrichment, the intermediates are coupled a
quantitatively.

Although various types of yeast couple pyrimidine an
azole, bakers' types are particularly efficient. Table 24 show
synthesis of thiamine from pyrimidine and thiazole by one
of bakers' yeast.[74] It will be noted that some synthesis occurr
adding thiazole alone. However, microorganisms vary
in their ability to respond to thiamine intermediates when
one is supplied.[31,32]

The synthesis of thiamine from pyrimidine and thiaz
favored by aerobic growth conditions, a temperature of 20° to
and by the presence of unidentified substances in various n
products.

21. SYNTHESIS OF THIAMINE FROM PYRIMIDINE AND THIAZOLE BY BAKERS' YEAST

Intermediate added to the medium			
yrimidine,[a] illimoles per liter	Thiazole,[b] millimoles per liter	Yield of dry yeast, grams per liter	Thiamine content, of dry cells, micrograms per gram
0.000	0.000	7.27	65
0.005	0.005	7.60	261
0.010	0.010	7.62	430
0.015	0.015	7.55	667
0.020	0.020	7.60	802
0.050	0.050	7.67	835
0.100	0.100	7.77	795
0.000	0.020	7.80	150
0.020	0.000	7.27	62

ethyl-5-ethoxymethyl-6-aminopyrimidine.
ethyl-5-β-hydroxyethylthiazole.

arly studies with pantothenic-acid-deficient yeasts showed that
ine promoted their growth and suggested that this compound
precursor of pantothenic acid.[78] This was confirmed sub-
tly by a number of investigators working with several dif-
types of organisms. One process by which yeast is enriched
pantothenic acid by adding β-alanine to the medium was
ed.[81]

dustrial applications to date have been confined to pre-
for thiamine and pantothenic acid and conjugated forms
tothenic acid. However, the same general technique has
adopted extensively to determine the pathway and inter-
es involved in the synthesis of other vitamins (folic acid,
niacin, etc.).

IONS OF CULTIVATION

ghly aerobic conditions, such as result from combined air
g and agitation, favor conversion of carbohydrate to yeast
bstance. Accordingly, per unit of carbohydrate utilized,
conditions result in a greater yield of vitamins. However,
m conditions for attaining maximum vitamin potencies vary
ach factor. For example, Singh, Agarwal, and Peterson[84]
strated with three species of yeasts that thiamine and ribo-
otencies decreased as aeration and agitation increased. Nia-
wever, increased directly with improved aeration, while folic
uld not be correlated with aeration rates.

SPECIES DIFFERENCES

Species variations become apparent when cultures are grown under similar conditions. This is shown in Table 25 in which results are given for the vitamin content of four species cultivate on identical substrates.[64] It may be seen that species differences i thiamine, niacin, and folic acid potencies are most pronounced Similar observations in the folic acid content of a number o yeasts have been reported by MacKenzie, Noble, and Peppler.[3 In this study, *Candida krusei* attained an unusually high folic aci content.

TABLE 25. VITAMIN SYNTHESIS BY DIFFERENT SPECIE OF YEASTS CULTIVATED UNDER SIMILAR CONDITION

Yeast	Yield based on sugar fermented %	*Vitamin content, micrograms per gra of dry cells*			
		Thiamine	Riboflavin	Niacin	Folic ac
Beet molasses					
S. cerevisiae	41	37	44	296	34
T. utilis	72	26	53	213	10
C. arborea	74	22	58	301	12
O. lactis	74	14	40	196	12
Cane molasses					
S. cerevisiae	38	28	45	402	34
T. utilis	74	21	54	282	15
C. arborea	71	16	60	313	20
O. lactis	69	12	41	195	15

These investigations emphasize the importance of selecti both the proper organism and proper operating conditions to a sure the maximum production of any desired vitamin by yeast.

Vitamin Synthesis in Other Industrial Fermentations

For many years, it was common practice to discharge the b of fermentation wastes into lakes and streams with only sm amounts being fed to livestock, largely in wet form. As the bre ing and distilling industries were expanded in the middle thirt this method of waste disposal caused serious water-polluti problems and eventually it became necessary to impose preven tive regulations. As a consequence of these restrictions, vari means of utilizing fermentation wastes were explored, includ a thorough investigation of their general nutritive value and c nt of individual vitamins. The second phase of this study was eatly facilitated by the development of microbiological-assay rocedures. The high nutritive value of residues from grain dis-llation and acetone-butanol production, which are now fully ecognized, was indicated by these investigations. These residues re currently recovered in dry form and marketed as feed supple-ents. The chemical composition of various fermentation wastes, ethods of recovery, and the development of the distillers' by-roduct feed industry have been outlined by Boruff.[9]

The vitamin content of fermentation residues is a reflection of a) the amount of fermentation products and volatile by-products emoved during fermentation and recovery and (b) the actual nthesis of vitamins which has occurred during the fermentation rocess. For example, in the ethanol fermentation of grain, there approximately a threefold concentration of residual matter rough the production of carbon dioxide and the removal of al-ohol by distillation. This concentration, coupled with the synthe-s of vitamins, of which riboflavin, pantothenic acid, and choline re outstanding, accounts for the high nutritive value of distillers' y-products. The vitamin contents of fermentation residues are hown in Table 26. Distillers' Dried Solubles represent the more luble fraction separated from the whole residue by screening or creening followed by centrifugation. Distillers' Dried Grains are he coarse fraction, which is termed Distillers' Light Grains, if dried irectly, and Distillers' Dark Grains, if mixed with the solubles raction after evaporation to 30 to 35 percent solids. It will be oted from the table that Distillers' Dried Solubles are higher in itamin content.

During the acetone-butanol fermentation, riboflavin and antothenic acid syntheses are appreciable, with the content of the irst being increased about tenfold over that initially present. yridoxine and pantothenic acid are also formed in this fermenta-ion. These are recovered by evaporating and drying the whole esidue.

In the commercial penicillin fermentation in which members f the *Penicillium notatum-chrysogenum* group are employed, there is a marked synthesis of pantothenic acid (about 18 μg per ml) and n increase in biotin and pyridoxine.[67] On a dry basis, the residues rom this fermentation are comparable with, or slightly higher in itamin potency than, Distillers' Dried Solubles. The dried mycelium

TABLE 26. VITAMIN CONTENT OF FERMENTATION RESIDUES[a]

Fermentation process	Material fermented	Type of residue	Thiamine	Riboflavin	Pantothenic acid	Niacin	Pyridoxine	Biotin	Choline
Ethanol	Corn-malt	Distillers' Dried Solubles	6-9	15-20	29-36	104-160	8-10	0.3-07	6,000-7,000
Ethanol	Corn-malt	Distillers' Dark Grains	3-4	7-10	12-15	70-90	4-6	0.2-0.3	4,000-5,000
Ethanol	Molasses	Condensed solubles[b]	—	5-7	5-25	40	25	1.0-1.5	—
Butanol	Molasses	Whole residue	15	60	300	—	30	—	—
Penicillin	Corn steep -lactose	Mycelium	3-7	20-48	88-212	107-180	21-25	0.6-1.5	3,000-4,000
Penicillin	Corn steep -lactose	Mycelium-free liquor	—	7-29	592-805	232-333	82-114	0.6-0.8	—
Butanediol									
A. polymyxa	Wheat	Whole residue	6	—	153	—	—	—	—
A. aerogenes	Wheat,	Whole residue	19	19[c]	121	—	—	—	—
	molasses, and corn		23-37	45-132	—	—	—	—	—

---

[a] μg per g, dry basis.

[b] A sirup of 40 to 45% solids.

[c] Partial inactivation during product recovery.

s been found nontoxic to poultry[38] and is being recovered by me producers as a feed supplement. The synthesis of relatively gh levels of pantothenic acid appears to be characteristic of the nicillia since other species including *P. utricae, P. intricatum,* d *P. stoloniferum* also produce unusual amounts.[68]

Two bound forms of pantothenic acid, i.e., coenzyme A and the *ctobacillus bulgaricus* factor (LBF), have been found widely stributed in microorganisms. Some commercial fermentation oducts are excellent sources and have been used to prepare puri- d preparations for identification and investigation purposes. enzyme A has been obtained from *S. fradiae* (neomycin) fermen- tions[40a] and is currently being recovered from brewers yeast.[4a] BF, a component of the coenzyme A molecule, whose reduced rm has been termed pantetheine [N-(pantothenyl)-2-amino- hanethiol][65b] is produced in high potency by *A. gossypii*[47a,65] and *subtilis*[75a] and has been purified from both sources.

Riboflavin, pantothenic acid, and probably other B vitamins e synthesized during the aerobic (*Aerobacter aerogenes*) and aerobic (*Bacillus polymyxa*) 2,3-butanediol fermentations.[1,11] *aerogenes* has been found particularly active in grain mashes in hich it produced as much as 9.3 μg of riboflavin per ml. This ganism has also been successfully applied to the fermentation of rain stillage in order to increase its vitamin potency.[40]

The formation of vitamins during other fermentations, such the production of organic acids, has not been extensively studied. owever, biotin[56] and rhizopterin, a derivative of folic acid,[83] have een found in media fermented to fumaric acid with *Rhizopus* ecies. Except for vitamin B_{12}, which will be discussed later, little formation is available on vitamin syntheses accompanying re- ntly developed antibiotic processes.

A few bacteria and molds, especially *Bacillus prodigiosus* and *spergillus niger,* have been observed to produce ascorbic acid.[75] ong incubation periods are usually required and the yields are arkedly affected by the conditions of cultivation. The highest eld reported is approximately 14 mg per 100 ml of medium.

MICROORGANISMS IN THE DISCOVERY AND PRODUCTION OF NEW VITAMINS

The requirement by both animals and microorganisms for

accessory growth substances was established toward the end of th
last century. Those factors required for animals were terme
"vitamines" by Funk[17] in 1912 and the name "bios" was applie
some years earlier by Wildiers[80] to the principle required for th
growth of yeast. For many years, investigations of the isolation an
characterization of these substances were continued along paralle
but completely independent lines. During this period, vitamins A
B_1, B_2, C, D, E, and K were elucidated and certain bios con
stituents, such as inositol, were isolated and identified. Althoug
Williams[81] had reported, as early as 1919, that yeast growth wa
stimulated by vitamin B and suggested that this might form th
basis for a vitamin assay, the microbiological approach was n
pursued until many years later.

Begining about 1930, a number of investigations disclose
that microorganisms required vitamins for growth and, conversel
that animals responded to certain microbial growth factors. Wi
liams and Roehm[82] observed that thiamine was required for t
growth of certain yeasts and Knight[30] showed that niacin, the ant
pellagra vitamin, was essential for the development of *Staphyl*
coccus aureus. Similarly, biotin, a growth-promoting substance f
yeast, was found to be identical with Vitamin H, the anti egg whi
injury principle. Subsequently, pantothenic acid and folic aci
which had been recognized earlier as microbial growth factors, we
demonstrated to have vitamin properties. These developmen
served to emphasize the close relationship between the various fiel
of nutrition, with the result that a general biological significance
implied for any new factor regardless of the original test speci

Recent studies on the nutritional requirements of vario
bacteria, yeasts, and molds have revealed the existence of a numb
of new growth substances. Some of these are finding practic
applications in nutrition. Indeed, it appears that required nut
ents brought to light through the study of microbial nutrition m
constitute the principal source of new vitamins. The sequence
utilizing microorganisms for this purpose has followed a rath
well-established pattern which involves the following steps:

(1) A particular microorganism is found to require (eith
naturally or following a mutation) a previously unidentified su
stance for growth or a specific biochemical function. This perm
the development of an assay procedure and the evaluation
natural products for potency.

(2) Not uncommonly, other microorganisms which do not re- uire the factor are found to synthesize it, and some in quantity, hus making available material for chemical fractionation.

(3) By the use of microbially synthesized substances, broad nu- ritional studies to establish vitamin activity are conducted with arious species and different types of rations.

(4) Final purification and determination of structure are arried out with microbial substances. Then, the vitamin can be repared commercially by either chemical or biological methods, epending on the relative cost.

This application of microorganisms to problems of animal utrition is well exemplified in the elucidation of the anti pernicious nemia factor (APA). This procedure was also used to good ad- antage in the study of folic acid[27] and the conjugated forms of antothenic acid.

Pernicious anemia is a deficiency disease characterized by efective erythrocyte formation. Liver for many years was the con- entional source of the curative factor and by clinical assay pro- edures, purification had proceeded to the point where as little as mg of concentrate represented a daily curative dose. However, urification and standardization of potency were tedious, since they vere dependent on the use of human patients during regression rom anemia. With the discovery of Shorb[62] that *Lactobacillus actis* Dorner responded to liver extracts in proportion to their PA activity, a rapid assay procedure was made possible and puri- ication of the active substance soon followed.[48]

This compound, vitamin B_{12}, is one of a family of complex oordination compounds containing trivalent cobalt which have een termed cobalamins.[65a] Vitamin B_{12} itself contains a cyano group in coordination with cobalt (cyanocobalamin) and has an pproximate empirical formula[49] of $C_{61-64}H_{86-92}N_{14}O_{13}PCo$. At least hree members of the vitamin B_{12} family have been found in micro- rganisms. A few micrograms of vitamin B_{12} elicit a positive ematological response in pernicious anemia patients. Nutrition tudies with animals have shown that this vitamin is essential for nimal growth and must be added to diets lacking in animal pro- tein. Vitamin B_{12}, therefore, is an important constituent of the "animal protein factor" (APF).

Several observations suggest that the microbiological synthesis of vitamin B_{12} is a significant factor in satisfying the requirement of

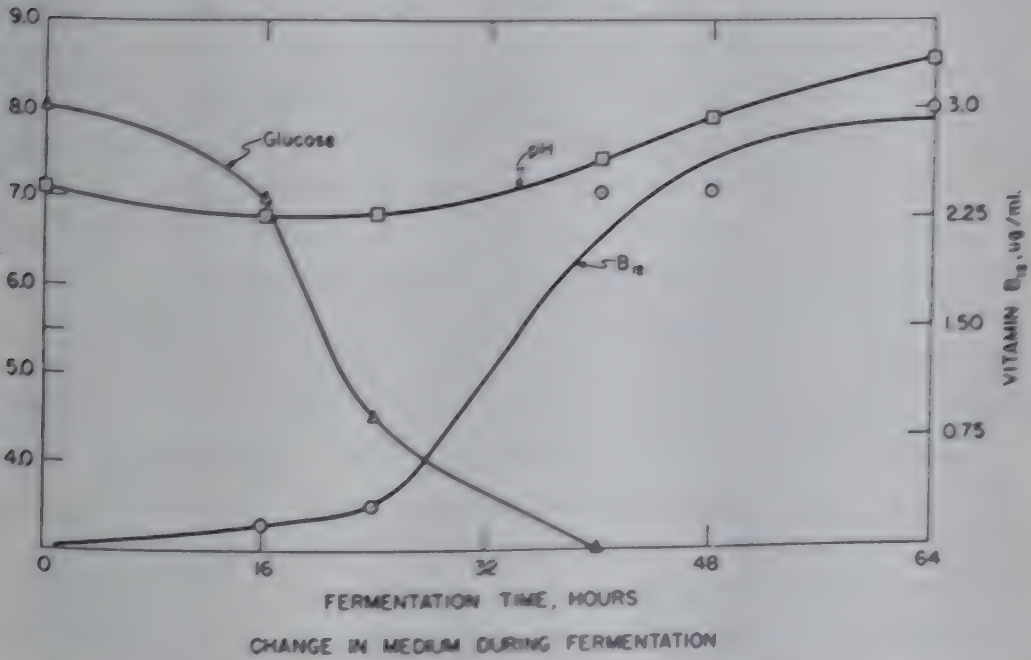
TABLE 27. QUANTITATIVE SYNTHESIS OF VITAMIN B_{12} BY VARIOUS MICROORGANISMS

Organism	Medium	Highest vitamin B_{12} potency reported μg/ml	References
Flavobacterium devorans	Glucose, soybean meal, corn steep liquor, cobalt and other inorganic salts	0.6	22[a]
Flavobacterium solare	Yeast extract, malt extract, glucose, salts or maltose, penicillin-mash residue	0.6	45[a], 46
Pseudomonas sp. and Proteus sp.	Soybean meal or other protein materials	0.4	25[a]
Saccharomyces cerevisiae and *Aerobacter cloacae*	Grain-ethanol stillage or penicillin-fermentation residues, inorganic salts	—	2, 13[b]
Bacillus megatherium	Beet molasses, ammonium phosphate, cobalt and other inorganic salts	0.45	18, 18[a]
Propionobacter freudenreichii	Glucose, N-Z-Amine, yeast extract, lactic acid, cobalt	3.0	32[a]
Streptomyces fradiae	Glucose, brewers' yeast, soybean meal, cobalt and other inorganic salts	0.7	37
Streptomyces griseus	Beef extract, N-Z-Amine, cobalt or glucose, soybean meal, distillers' solubles, cobalt	0.3	25, 83[a]
Streptomyces olivaceus	Glucose, distillers' solubles, cobalt or soybean meal, cobalt and other inorganic salts	3.3	20[b], 21
Streptomyces vitaminicus	Glucose, animal stick liquor or other protein	2.0	[illegible]

ls for this vitamin. Rubin and Bird[22] found cow manure to good source of the factor probably owing to rumen and in- l synthesis. Chicken feces, although low in the factor when l, also acquired activity on incubation at temperatures favor- icterial growth. Substantiation of microbial synthesis was ed by Stokstad and associates[66] with the finding that a bac- isolate from chick feces synthesized the factor in media of composition.

Vithin the past few years, microorganisms have been intensively ed for their ability to biosynthesize vitamin B_{12}.[9b,20a,22,52a,63,71] istribution of this vitamin in fungi and plant tissues has been bject of a recent review.[13a] The surveys have revealed that a percentage of bacteria and actinomycetes, but only a few yeasts filamentous fungi produce vitamin B_{12}. Table 27 lists organisms which synthesize amounts sufficient to receive ercial consideration.

urrent requirements for pharmaceutical- and feed-grade in B_{12} are being met by primary fermentation methods and covery as a by-product of certain antibiotic fermentations, streptomycin, aureomycin, and terramycin fermentations. ner potential source is activated sludge from municipal



8. *Course of Vitamin B_{12} Fermentation with* Streptomyces olivaceus [Reprinted by permission from *J. Appl. Microbiology,* **1,** 124 (1953)]

sewage-treatment systems.[55a] Of the primary fermentations
Streptomyces olivaceus has been used widely, particularly i
manufacture of animal feed supplements. Figure 8 from the
by Hall et al.[41] shows the course of a typical deep-tank fe
tion with this organism. Cobalt, a component of the vitar
molecule, is present in inadequate amounts in the usual fe
tion substrates and must be added for maximum biosynthes
fortification of media with both cobalt[55a] and cyanide[54a] h
patented. Vitamin B_{12} is retained within the cells of certain
which produce it[15a,58a] and it is absorbed by other species
property offers a method of concentration. The chemistry
min B_{12}, its purification from natural sources, and its role in
nutrition have been discussed in detail elsewhere.[60a,83]

FAT-SOLUBLE VITAMINS AND PROVITAMIN

The fat-soluble vitamins and provitamins, in contrast
B complex vitamins, are sporadically distributed among th
Ergosterol (provitamin D) occurs widely in yeasts and mo
in certain bacteria. The carotenoids, of which several are p
or wholly convertible to vitamin A in the animal body,
duced by a number of chromogenic bacteria[19a,75] as wel
representatives of the following genera of yeasts and
Saccharomyces, Rhodotorula, Sporobolomyces, Mucor, and
mycetes. Vitamin K (K_2) is limited to certain lactics and c
genes organisms, while vitamin E is not an established con
of fungi. It should be pointed out, however, that the fat
factors are not uncommonly produced in relatively large
by fungi. For example, ergosterol[6] may comprise as much
and carotene[57] as much as 0.25% of the dry-cell substanc
tain species. Nevertheless, except for ergosterol, which wi
cussed later, no concerted effort has been made to exp
industrial aspects of microbiologically synthesizing fa
vitamins, since these substances are more economically
from other natural and synthetic sources. Several books
views contain discussions on the distribution and significan
fat-soluble vitamins and related compounds in fungi.[6,19a]

Ergosterol

Ergosterol, which is the principal sterol of fungi.

from ergot by Tanret[69] in 1889. In the years immediately
g, it was observed to be widely present among various
ganisms. Interest in this substance was greatly stimulated
discovery, in 1927, that it became antirachitic on irradia-
ssays of various natural products for ergosterol potency
that yeasts and molds were exceptionally rich sources. The
g values, taken from the literature, show the ranges which
n reported for the three general groups of microorganisms:
0 to 0.49%, yeasts 0.14 to 2.9%, and molds 0.02 to 2.3%.
s evident that certain yeasts and molds offer commercial
lities as ergosterol sources, provided they can be econom-
ropagated or recovered as by-products of other fermenta-
Originally, the mycelium produced in citric acid manu-
and brewers' yeast were processed. These have been
ted for the most part by special methods of yeast propaga-
ich result in products of higher ergosterol content. In ad-
some attention has been given recently to the ergosterol
of the mycelium of molds produced in antibiotic fermen-
particularly penicillin, which might be utilized under
circumstances. The utilization of various sterols as inter-
s in cortisone production has renewed interest in the
d amounts of these compounds found in fungi.

diated ergosterol (vitamin D_2, calciferol) is of low activity
chick, but is highly active for mammals. It is used ex-
in foods and pharmaceuticals and in feed supplements
-footed animals. The production of this vitamin in the
States is shown in Table 28.

8. PRODUCTION OF IRRADIATED ERGOSTEROL IN THE UNITED STATES

Year	U.S.P. units $\times 10^9$
1941	728[a]
1942	5,892
1943	14,248
1944	33,906
1945	27,301
1946	33,448
1947	15,610
1950	30,346
1952	25,447

illion units per lb.
U. S. Tariff Commission.

The ergosterol content of microorganisms is a functior the particular species or strain employed, (b) the medium c tion, and (c) the physicochemical environment. Several r for increasing ergosterol synthesis, based on one or more features, have been developed and patented. Current p although never fully disclosed, probably represent combina exceptional yeast strains in conjunction with favorable and chemical conditions.

SPECIES AND STRAIN VARIATIONS

When cultivated under similar conditions, both ye molds may vary widely in ergosterol content. Bills, Ma and Prickett[8] found, for example, that in twenty-nine y amined, the ergosterol content ranged from a trace w *charomyces logos* to 2% with *S. carlsbergensis.* Similarly, isolates from one species varied from 0.2 to 1.4%. Varia similar magnitude have also been observed with numerou Because of its characteristically high ergosterol content, *bergensis* is probably the species most widely adopted comn

PHYSICOCHEMICAL CONDITIONS

Several physicochemical factors have been explored effect on ergosterol synthesis by yeasts. Of these the mos is the degree of aerobiosis. The marked effect of aeration noted by Bills, Massengale, and Prickett,[8] who found that yeast, with only a trace of ergosterol, contained 1.4% whe gated aerobically. Likewise, Bennett[5] has observed tha nontoxic oxidizing agents, such as peroxides, persulfates, bined with aeration and a reduced nitrogen supply, fu hanced ergosterol formation. These modifications, in ad supplementing the medium with ethanol and incubat higher temperature during the latter part of the ferment claimed[6] to raise the ergosterol level to as much as 2.7% and slightly alkaline conditions also have been reported fa ergosterol formation.

MEDIA

In view of the lipid nature of sterols, it has been that conditions which promote the formation of fats mig pected to increase sterol synthesis. However, attempts

uction of total lipid to that of sterols have been largely
ful. More commonly, the relationship is an inverse one.
easts, at least, a correlation has been noted between sterol
and the particular type of carbohydrate supplied, di- and
rides yielding more sterol than monosaccharides.[34] As
id has also been observed to enhance ergosterol synthesis
. With *Aspergillus fischeri,*[22] 10% glucose, a glucose-urea
20:1 and an initial pH of 2 were found most favorable.
nentioned before, commercial ergosterol originates mainly
asts, but might also be derived from the by-product
n of mold fermentations. Examples are the mycelium of
um notatum,*[23,24] *Aspergillus flavus-oryzae* cultured on
an, and, as stated previously, *Aspergillus niger* from the
on of citric acid. *P. notatum,* however, varies widely in
depending on the particular strain and the conditions of
ion. For example, mycelium produced under submerged
ns contained only 0.22%,[33] while that grown on the surface
itated media yielded 2.3% when incubated for several
Thus, the submerged method of penicillin production re-
mycelium of low potency, but the variability between
ffers some possibility of selecting more active organisms.
conversion of ergosterol to vitamin D is carried out at
of several stages, depending on the final use of the
d product. Yeast cells, after centrifuging and washing,
nonly irradiated directly with ultraviolet light and then
nd dried if to be used in feeds. For pharmaceuticals and
tification, the cellular material is first autolyzed or
d and then saponified with alkali.[19] Ergosterol is then
with ether and further purified. This purified product
ted in suitable solvents, such as benzene or xylene. Rosen-
s discussed in some detail the irradiation of ergosterol
the chemistry and physiology of the activated compound.

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PART V. THE PRODUCTION OF PHARMACEUTICALS

APTER 7

THE PENICILLIN FERMENTATION

John C. Sylvester and Robert D. Coghill

INTRODUCTION

Brief History

Penicillin is the name applied by Fleming[23] to the bacterio-
tic principle produced by a mold which was later identified as
nicillium notatum. Since Fleming's discovery, in 1929, it has
en found that penicillin is produced by a variety of molds be-
ging to other species and genera and also that there is a series
closely related penicillins, all of which have approximately the
ne antibiotic characteristics. The word penicillin has, there-
e, become a generic term which can be properly applied to a
ge number of closely related chemical entities.

Following Fleming's original work, Clutterbuck, Lovell, and
istrick[15] undertook to study the chemistry of penicillin. The
ults of their work, published in 1932, indicated that penicillin
s an organic acid which was extractable into organic solvents
m aqueous solutions of low pH, but that it was extremely
ile to hydrogen ion, heat, and even disappeared on evaporation
a solution to dryness. In view of this great lability and the
y low yields (less than 1% of current practice), work on peni-

cillin was abandoned in favor of more promising projects. Afte
this, except for a paper by Reid[42] in 1935, nothing further ap
peared on penicillin for a period of 8 years.

In 1940, stimulated by the urgent need for better agents fo
the treatment of burns and other wounds, Chain and Florey,
with their collaborators, undertook the reinvestigation of peni
cillin. They cultured Fleming's organism in surface culture o
what amounted to a small pilot-plant scale. By keeping a lo
temperature during their extraction procedure, they were able t
concentrate the penicillin a thousandfold and produce a dry pow
der, in the form of a salt of penicillin, which had reasonabl
stability on storage. This represented the first great advance i
penicillin production and furnished the material which was use
to demonstrate the phenomenal curative properties of th
antibiotic.

Because of the virtual impossibility of quickly producing
significant amount of penicillin under wartime conditions i
England, Florey and Heatley came to the United States in Ju
of 1941 in order to enlist the aid of the American government ar
pharmaceutical industry. The story[16,17] of their success in th
direction has been told before and there is no need to repeat
here. Suffice it to say that by the time of the invasion of Fran
in 1944, adequate amounts of penicillin were available on a
fronts to effect a tremendous saving of life among the wounde
Penicillin was most certainly one of the few benefits accrui
from World War II. Had it not been for the urgent need f
such a curative agent, for the wartime ease of securing gover
ment money for such research projects, and for the excess prof
tax which made it possible for industry to make a terriffic gaml
with fifteen-cent dollars, we might not have had penicillin ev
today.

Therapeutic Usefulness

Penicillin is without doubt the most outstanding drug e
discovered for the treatment of infections. Its high order of
tivity, combined with its virtual nontoxicity, serves to make
the drug of choice for the treatment of most conditions cau
by susceptible organisms. Its only bad feature is the fact that

oximately 5% of those receiving the drug may suffer from some gree of allergic response. This condition, however, can fre- uently be averted by the use of a suitable antihistaminic drug.

Penicillin is active, in general, against Gram-positive micro- ganisms and inactive, with certain notable exceptions, against ram-negative forms. Although occasional resistant strains are ncountered, penicillin is markedly effective against pathogenic rains of the following genera: *Micrococcus, Streptococcus, iplococcus, Neisseria, Clostridium, Treponema, Borrelia, Lepto- ira, Corynebacterium, Bacillus,* and *Actinomyces.* In addition, rtain strains of other types of microorganisms, including some of e larger viruses and *Rickettsia* (such as *Miyagawanella psit- cii),* are susceptible. In general, penicillin is ineffective against ch genera as *Escherichia, Aerobacter, Klebsiella, Proteus, Sal- onella, Shigella, Pasteurella, Eberthella, Pseudomonas, Vibrio, rucella, Hemophilus, Mycobacterium, Rickettsia,* and yeasts, molds, nd viruses.

Although the previous lists are incomplete, they give an ap- roximate idea of the range of effectiveness of penicillin and serve indicate its wide area of applicability—including many fields which there has before been no completely satisfactory remedy. he necessary dosages of penicillin vary greatly from the 50,000- nit, single-dose treatment of gonorrhea to the several million nits per day over a long period of time necessary in the treat- ent of subacute bacterial endocarditis.

Penicillin may be used topically, orally, or parenterally. When sed orally, the dosage must be much larger (at least fivefold) ecause of destruction in the digestive tract as well as incomplete nd variable absorption. When used parenterally, the intra- uscular route is the usual method of choice.

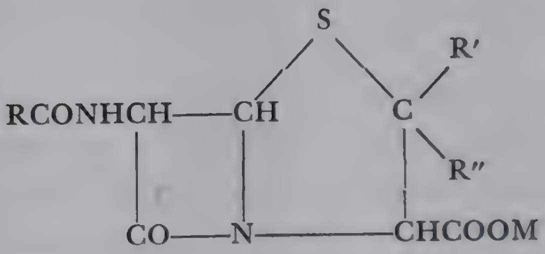
Various salts of penicillin are in current use. The potassium, odium, and calcium salts are all highly water soluble and can be sed interchangeably. The calcium salt has not yet been crystallized nd is available only in the crude, amorphous form. The crystal- ine potassium salt of penicillin G will undoubtedly attain the idest distribution among all soluble penicillin salts because of ower manufacturing costs as compared with the crystalline sodium alt or any of the amorphous forms. It is an example of a curious situation that a relatively pure crystalline substance is cheaper to produce than the impure amorphous compound.

More recently, the insoluble procaine salt of penicillin G has achieved popularity in repository dosage forms which permit the injection of penicillin at much less frequent intervals.

The Chemistry of Penicillin

The chemistry of penicillin has been discussed very comprehensively in a recent monograph.[14] In this chapter, only those phases of the subject will be covered which are essential to a proper understanding of the problems arising in penicillin production by fermentation.

Penicillin, as mentioned before, is a generic term which refers to a class of compounds, the known members of which have the structure:

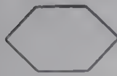
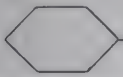


Penicillin

where R may be a simple substituted alkyl or aryl group, R′ and R″ are simple alkyl groups, and M is a hydrogen atom or an equivalent of base (organic or inorganic). In all of the known mold-produced penicillins, R′ and R″ are methyl groups and the M depends on the method of isolation. The chemical nomenclature in current use assumes that R′ and R″ are methyl groups, the distinctive name of a particular penicillin being derived from the nature of the R group. When the R is the benzyl group ($C_6H_5CH_2$—), the specific name becomes benzylpenicillin and the sodium salt is called sodium benzylpenicillinate. There is, however, another system of nomenclature which has a historical rather than a chemical background, but which is the only system used in the penicillin trade. Under this system, benzylpenicillin is termed penicillin G and its sodium salt, sodium penicillin G.

The common penicillins known to be produced by fermentation are listed in Table 29, along with their designations under both systems of nomenclature. As this chapter is designed primarily for those interested in producing penicillin by fermentation, only the trade terminology will be used.

TABLE 29. PENICILLIN TERMINOLOGY

R	Chemical Name	Trade Name
$CH_3CH_2CH=CHCH_2$-	2-Pentenylpenicillin	Penicillin F
$CH_3(CH_2)_3CH_2$-	*n*-Pentylpenicillin	Dihydropenicillin F
$CH_3(CH_2)_5CH_2$-	*n*-Heptylpenicillin	Penicillin K
 —CH_2-	Benzylpenicillin	Penicillin G
HO  —CH_2-	*p*-Hydroxybenzylpenicillin	Penicillin X

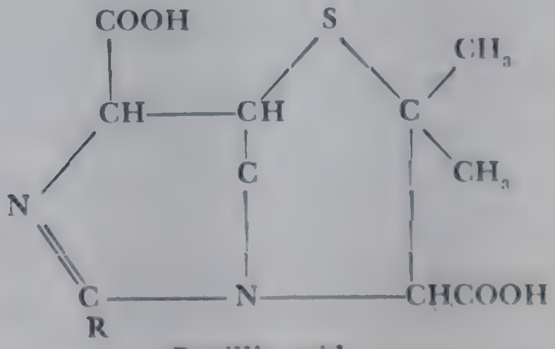
Penicillin measurement was originally accomplished by the so-called "cylinder plate method."[14,25] Based on this technique, an arbitrary unit, the Oxford unit, was established. In October of 1944, however, at an international meeting held in London, the International Unit was established. As defined there, an International Unit is that amount of penicillin which is equivalent in activity to 0.6 μg of the International Standard, as measured against either of two specified strains of *Staphylococcus aureus* (one of them being the C-203 strain of the United States Food and Drug Administration). The International Standard consisted of a sample of crystalline sodium penicillin G which was as pure as was possible to prepare at that time, and which, in retrospect, we know to have contained more than 98% sodium penicillin G.

The nature of the R group or, in other words, the type of penicillin produced, can be determined, to a large extent, by incorporating in the medium a precursor which may be a derivative (salt, amide, or ester) of the corresponding acid, RCH_2COOH, or an amine of the formula $RCH_2CH_2NH_2$. Not all compounds having these formulas will serve as precursors of the corresponding penicillins, but literally dozens of them have been so used. In the absence of an effective precursor, the type of penicillin produced seems to be a function of the mold strain and, in general, one obtains a mixture of several penicillins. Fleming's original strain of *P. notatum,* for instance, seems to have produced on his medium largely penicillin F. It is thus highly probable that this was the penicillin which he discovered and which the English workers subsequently isolated in the pure crystalline state. However, the currently used commercial strains, i.e., nonpigment-secretin descendents of *P. chrysogenum* Q176, when cultivated in the ab sence of precursors, produce a mixture of penicillins which con sists largely of penicillin K, along with smaller amounts o dihydropenicillin F.

Penicillin manufacturers, however, strive to produce pen cillin G solely, as this is the only penicillin in demand by th medical profession. This demand arises from the fact that pen cillin G has better pharmacological properties and stabilit than penicillins K, F, and dihydro F, and is cheaper to produc than penicillin X. Other penicillins, equally as good as G, coul probably be made cheaply and easily by the proper choice o precursors, but as they differ only *quantitatively,* rather tha *qualitatively,* in their effectiveness against pathogenic organism it is unlikely that anyone will ever try to overcome the tremendou inertia of the literature and medical practice which placed pen cillin G on the pedestal it occupies today.

Sodium penicillin G is the salt of a strong hydrophobic ac whose pK is 2.75. The free acid can, therefore, be extracted fro water into a wide variety of solvents when the pH of the solutic has a value of 2 to 3. The inorganic salts of penicillin are solub in water, methanol, and ethanol, but are insoluble in anhydro solvents, such as acetone, ether, chloroform, and esters. Additic of small amounts of water, however, greatly increases the solubili of penicillin salts in these solvents. Sodium penicillin G has specific rotation ($[\propto]_D^{20-25}$ in water) of approximately +300

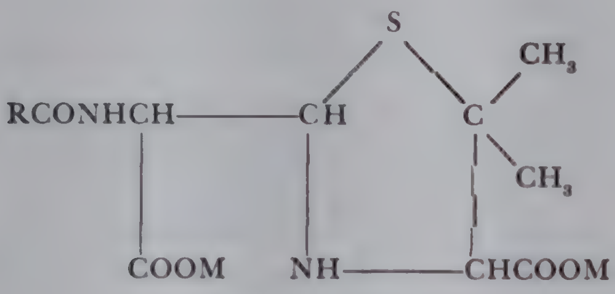
Penicillin is quite unstable in the presence of hydrogen io Under such circumstances, it rapidly rearranges to a biological inactive isomer, known as penillic acid, which has the followi structure:



Penillic acid

pH 2 to 3, this reaction goes almost to completion in 3 to 4 ours at room temperature. It is therefore necessary, in the isola- on of penicillin, to minimize the time during which it is ex- osed to low pH and to maintain as low a temperature as possible uring such exposure.

Penicillin is also very unstable at an alkaline reaction. At I 12 at room temperature, the β-lactam ring opens quickly to eld a salt of what is known as penicilloic acid, having the fol- wing formula:

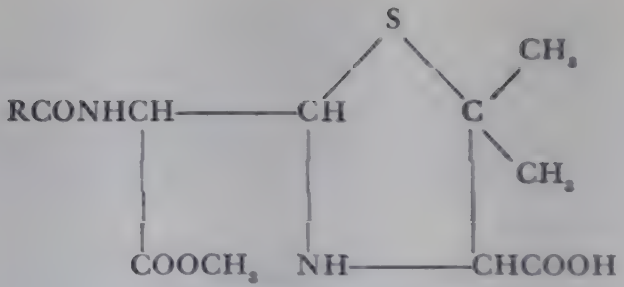


Penicilloic acid

or this reason, it is unwise to expose penicillin to a pH greater an 8. This reaction is catalyzed by many heavy metal ions, in articular Zn and Cu, and one must exercise every precaution to clude even faint traces of these ions from processing operations.

The same reaction as produced by alkali, in all probability, also brought about by exposure of penicillin to penicillinase, n enzyme commonly produced by many bacteria which are boratory and plant contaminants. At room temperature, in the eighborhood of neutrality, small amounts of this enzyme can uickly and completely inactivate tremendous quantities of peni- llin. Although the end product of the reaction has not been olated, the production of a new acidic and a new basic group, aving the same pKs as the corresponding groups in penicilloic cid, as well as other chemical evidence, leads strongly to this terpretation of the reaction.

Primary and secondary alcohols also lead to the inactivation f penicillin, methanol and ethanol being the worst in this re- pect. The reaction is again one of opening the β-lactam ring, the alf esters of the penicilloic acids having been isolated from the eactions. In the case of methanol, the inactivation product has e following structure:



α-Methyl penicilloate

In this reaction, also, heavy metal ions act as powerful cataly The higher primary alcohols, as well as secondary and terti alcohols, react in this manner at such a low rate that, with pro timing and temperature, it is possible to use them in penicil recovery operations.

A similar reaction with primary amines also leads quickly penicillin inactivation. The end products of this reaction are corresponding α-amides of the penicilloic acids. Presuma secondary amines will react in the same fashion at a lower r It is thus important, in making the salts of penicillin with orga bases, to use tertiary amines. An exception to this, however, been the procaine salt, where the weak basicity of the NH_2 gro presumably slows down the reaction, so that there is little inact tion before the insoluble salt has come out of solution.

Finally, one must consider the inactivation of penicillin heat. Amorphous penicillin, whether impure or very pure, is unstable to dry heat, 1 or 2 hours at 100°C leading to virtu complete inactivation. The nature of this reaction has not b clearly demonstrated, but it probably is a reaction with absor water. The dry crystalline sodium or potassium salts of the p cillins are quite heat stable; sodium penicillin G, for example completely stable at 100°C for as long as 30 days.

Economic Considerations

The economic importance of penicillin far exceeds that of other product of the pharmaceutical industry and also of fermentation industry, with the possible exception of the alco The United States Tariff Commission has reported that Un States penicillin production for 1946 and 1947 was valued at proximately $90,000,000 per year and for 1948 at $137,652. The value for 1951 was about $137,517,000. No other phar ceutical has ever approached these figures. Of this amount, a $50,000,000 worth is exported annually (as of 1951). Exp

1952 were lower because of much lower prices and foreign duction.

The world's production of penicillin is centered almost en- y in the United States with the exception of England, where duction is about 10% of that of the United States. No other ntry approaches even 1% of our capacity. It is true, however, modern penicillin plants are being built in many countries the United States will probably not be able to maintain its ent large exports which support production to a considerable nt.

Table 30 gives the United States production[46] of penicillin e its inception in 1943, as compiled by the United States De- tment of Commerce, except where otherwise noted.

TABLE 30. PRODUCTION OF PENICILLIN IN THE UNITED STATES

Year	Units × 10^9
1943	21
1944	1,633
1945	7,125
1946	25,809
1947	30,640
1948	90,501
1949[a]	138,100
1950[b]	219,903
1951[a]	324,293

From U. S. Tariff Commission.
Chem. Eng. News, **29,** 3050 (1951).

Penicillin prices have followed the usual pattern of increas- production. Table 31 gives the prices of vials of 100,000 units penicillin which have been certified for parenteral injection.

TABLE 31. PRICES OF PENICILLIN

Date	Wholesale price per 100,000 unit vial[a]
July 1943	$20.00
October 1943	15.00
January 1944	7.00
April 1944	3.75
July 1944	2.75
April 1945	0.78
January 1946	0.51
January 1947	0.36
January 1948	0.31
January 1949	0.20
January 1950	0.13
January 1951	0.15
January 1952	0.15
June 1952	0.13

Through April 1945, this represents the price on government contracts, as penicillin was sold to civilians.

These data depict a truly remarkable happening. In a perio of 5½ years, the price of penicillin has dropped to less than 1% of its first quoted price and, during that time, the quality im proved from that of a relatively unstable amorphous powde containing 10 to 20% of penicillin to that of a stable crystallin salt of penicillin G of 90 to 99% purity.

Bulk penicillin has had an equally precipitous drop in pric and, as of November 1953, the following prices[5] per million unit (in large quantities, bulk) prevailed: Crystalline potassium penicillin G, sterile, 19 to 22.5¢, crystalline procaine penicillin G sterile, 18 to 20¢.

Crystalline potassium penicillin G at 4¢ per 100,000 unit represents a price of approximately 64¢ per gram. Assuming al penicillin to have been of this quality and price, the United State production for 1950 had a weight of approximately 300,000 lb an a value of $90,000,000. When one considers that the major po tion was sold as higher-priced procaine penicillin and that th price of the many dosage forms (tablets, oil suspensions, inhal tion powders, etc.) varies from two to five times the bulk pric it is evident that the penicillin business at the wholesale lev amounts to several hundred millions of dollars a year.

THE PENICILLIN-PRODUCING ORGANISMS

The mold described by Fleming,[23] in 1929, and subsequent identified as a strain of *Penicillium notatum* by Dr. Charles Tho of the United States Department of Agriculture, was used in t early developmental stages of the surface-culture process. The yiel with this culture were poor and considerable work was carrie out in several laboratories in an attempt to obtain a higher-yiel ing strain. Several strains,[34] including a descendant of Fleming culture, were found which produced higher yields of penicilli These new strains, together with other improvements in t process, combined to give yields of penicillin in the range of 2 units per ml by the surface-culture method. None of these cu tures, however, performed well in submerged culture.

Many strains of *P. notatum* and *P. chrysogenum* were test in an effort to find one which would produce good yields the submerged process. One of the first good cultures found w a strain of *P. notatum* NRRL 832 of the Northern Regional

rch Laboratory.[35] This strain was used by most producers dur-
g the early stages of the development of the submerged process
d was capable of producing in the range of 100 units of peni-
llin per ml.

Continued work at the Northern Regional Research Labora-
ry[39] resulted in the isolation of *P. chrysogenum* NRRL 1951,
hich gave somewhat higher yields than did the strain NRRL
2. By plating NRRL 1951, followed by selective isolation, strain
RRL 1951•B25 was obtained. This strain was significantly better
an the parent. Demerec,[20] at the Carnegie Institute, then sub-
cted NRRL 1951•B25 to X-ray irradiation and isolated the
rvivors. His strains were screened at the University of Min-
esota and further tested at the University of Wisconsin and one
them, X1612, was found to be significantly superior to the
rent NRRL 1951•B25. This culture was subjected to ultra-
olet irradiation by Backus and Stauffer[6] at the University of
isconsin and a strain designated Q176, which proved superior
X1612, was isolated. This strain was adopted by most of the
enicillin producers and monospore isolates from it are in gen-
al use.

Raper and Alexander[39] have reviewed very completely the
istory of the various strains of *Penicillia* that have been of
gnificance in the development of penicillin. Their paper also
cludes detailed descriptions of the strains and a discussion of
eir extreme variability and methods of maintaining stock cultures.

The penicillin-producing molds are characterized by unusual
ariability; in general, the greater the productivity of the strain
e more unstable it is. The extreme variability of these organisms
resents the microbiologist with a difficult problem in maintain-
g active stock cultures of consistent penicillin-producing capac-
y. This problem is of the utmost importance in commercial
roduction where the cost of the finished product varies almost
versely with the yield in the fermentors.

Stock cultures can be maintained as agar slants, in dry soil,
r in lyophilized form (see Chapter 12). Stocks carried on agar
ants are perhaps most liable to variation since successive trans-
r of the organism provides greater opportunity for variation
occur. Frequent transfers tend to propagate selectively those
ortions of the culture population that sporulate most readily
d profusely. If agar slants are used, transfers should be relatively

infrequent. A medium that provides minimum vegetative growth and maximum sporulation should be employed and large numbers of conidia should be used as inoculum. One company has found the preservation of stocks by freezing agar slant cultures, after good sporulation is obtained on the slants, to be very satisfactory

Soil stocks are prepared by adding a heavy aqueous suspension of spores to tubes of sterile soil, allowing the soil to dry at room temperature, and then storing at 2° to 4°C. Stocks carefully prepared in this fashion will keep indefinitely and it is very little work to prepare enough tubes at one time to last for many years. This procedure has proved satisfactory for the penicillin-producing molds in most cases. However, one instance is known in commercial production in which stocks of *P. chrysogenum* X1612 preserved on soil gradually deteriorated over a period of about 1 year to the point where yields of less than 10 units per ml were obtained from stocks which originally had produced several hundred units per ml. A similar experience in a research laboratory is reported by Gailey, Stefaniak, Olson, and Johnson.[24]

It is believed by many that lyophilized stocks are best for preserving penicillin-producing cultures. To prepare stocks, a mass of conidia is suspended in a small amount of whole blood serum, milk, or peptone solution. The spore suspension is then dispensed into small tubes, dried from the frozen state under high vacuum, and the tubes sealed. An excellent description of this technic is given by Raper and Alexander.[40] As in the case of soil stocks, sufficient tubes can be prepared at one time to last for many years.

The microbiologist charged with the maintenance of stock cultures of penicillin-producing molds should constantly keep in mind the instability of these organisms. He should be continually alert to morphological variation and deterioration in penicillin producing capacity. It may be necessary, when such changes occur, to isolate from the stock culture, by plating, a number of strains which are then tested. It is possible, in many instances to recover from a deteriorated stock strains which are equivalent to the original in penicillin-producing ability. Churchill and Roegner,[12] working under the direction of Backus and Stauffer have studied the population patterns and variability of *P. chrysogenum* Q176 and related strains. They describe five types of colonies and point out that all five types show instability. A wide varia

on in yield of single-spore isolates of *P. chrysogenum* Wis. Q176
described by Reese, Sanderson, Woodward, and Eisenberg.[41]

CHEMICAL CHANGES DURING THE FERMENTATION

While the production of penicillin may be considered a fer-
entation process, the product, penicillin, is not a true fermenta-
on product in the same sense as are gluconic acid, lactic acid,
lcohol, etc. True fermentation products are usually the result
f the energy metabolism of the cell and, as such, the
echanism of their formation is comparatively clearly defined.
enicillin and most other antibiotics, however, are apparently
ot directly related to the energy metabolism. In the lactic acid
rmentation, 90% of the sugar fermented may appear as lactic
cid; in contrast, in the penicillin fermentation, based on the
arbohydrate metabolized, a yield of only 3 to 5% of penicillin
obtained. Also, it is possible to obtain, with an otherwise
ormal fermentation, little or no penicillin. It seems likely that
enicillin results from the metabolism of the amino acids and
ther complex organic compounds when a natural medium is
sed. However, the mold can synthesize penicillin when grown in
ynthetic media containing only an energy source and inorganic
onstituents. The general opinion is that penicillin is a minor
y-product of the normal metabolism of the organism and plays no
ssential role in mold metabolism. This comment is applicable
o most antibiotics as well as to the excessive production of vita-
nins by certain organisms. The question of why and how peni-
illin is produced cannot be satisfactorily answered on the basis
f our present knowledge. It is hoped that the intensive studies
eing carried out on this fermentation will soon provide some
xplanation of the process.

The chemical changes which occur during the penicillin fer-
nentation vary widely, depending on the composition of the
nedium. While these variations are of the greatest interest from
research standpoint and lead toward an elucidation of the
nechanism of penicillin production, for the purpose of this re-
iew, it will suffice to consider only those changes which take
lace in the medium generally in use for commercial production.
Vhile the specific media employed by the various penicillin pro-
lucers are carefully guarded secrets, it is believed that all manu-

facturers employ variations of a similar formula composed primarily of the following substances:

	%
Corn-steep liquor solids	1-5
Lactose	1-3
Glucose	0-1
Sodium nitrate	0-0.5
Calcium carbonate	0-1
Phenylacetic acid or a derivative	0.01-0.1
Water	

While the exact composition of corn-steep liquor is unknown the proximate analysis,[8] on a dry basis, is as follows:

	%
Total solids	40-60
Total nitrogen	7.4-7.8
Amino nitrogen	2.6-3.3
Lactic acid	12-27
Reducing sugars (as glucose)	1.5-14
Ash	18-20

In addition to these ingredients, small amounts of phenylala nine, tyrosine, β-phenylethylamine, tyramine, and similar con pounds of significance in the penicillin fermentation are presen The concentrations of lactic acid and sugar will vary in differe lots of corn-steep liquor, depending on the amount of ferment tion that has taken place. In general, however, the amount sugar plus the amount of lactic acid is approximately constan While the composition of different batches of corn-steep liqu will vary, the components which are important for the ferment tion are present in adequate amounts in most lots. These su stances are lactic acid, organic nitrogen, precursors for penicill G and other penicillins, and mineral salts. The chemical chang of a corn-steep liquor medium during a typical fermentation *P. chrysogenum* Q176, as described by Brown and Peterson,[9] a shown in Figure 9. The chemical changes and the metabolism the penicillin-producing molds are reviewed very excellently papers by Peterson[37] and Johnson.[28]

Carbohydrate Utilization

A wide variety of carbohydrates can be utilized by *P. chryso num* for growth. The group of compounds which can provi

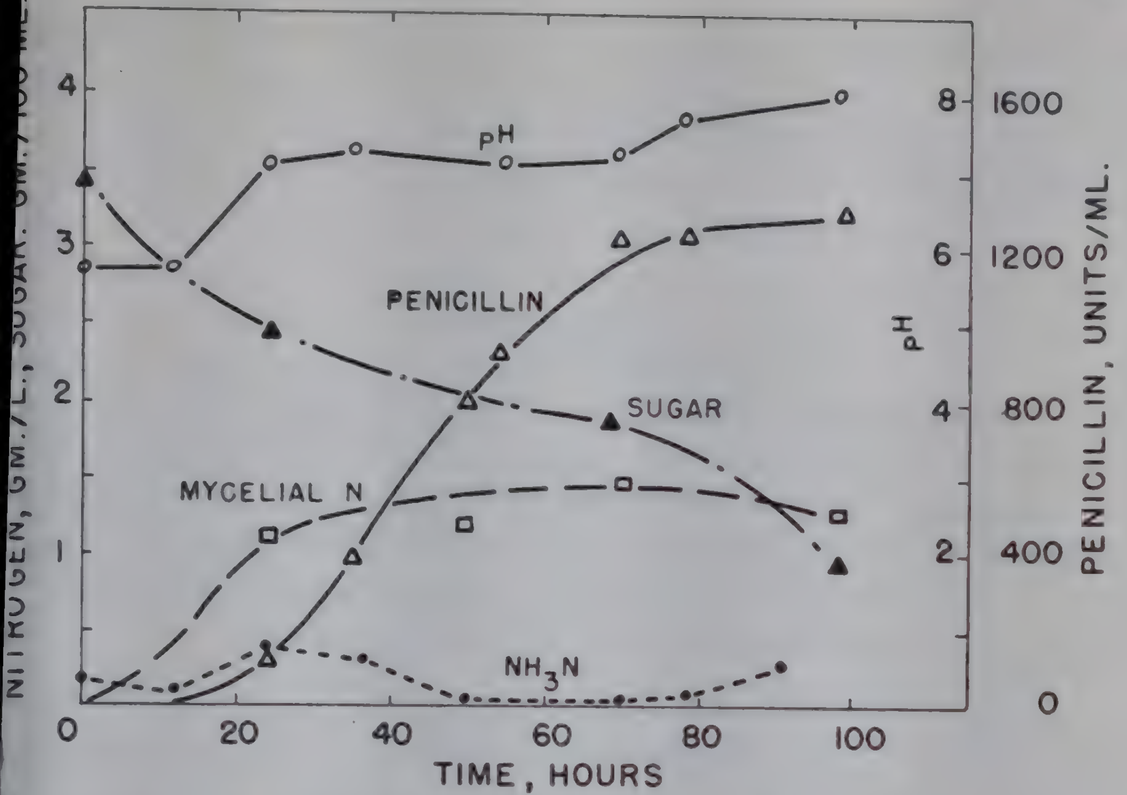


FIGURE 9. *Chemical Changes in a Typical Fermentation with Added Phenylacetic Acid*[9]

nergy and carbon includes various organic acids (such as lactic, cetic, and formic), amino acids, starch, dextrins, glucose, sucrose, rown sugar, lactose, and many other sugars. Lactose gives the ighest yields of penicillin and is generally used commercially. rown sugar, while somewhat inferior to lactose, is suitable for ommercial production. It is possible, under special conditions, s, for example, very high aeration in glucose media, to obtain ood yields on carbohydrates other than lactose. However, such ecial conditions are not suitable for industrial application. Re-ently Hosler and Johnson,[27a] employing a basal synthetic medium, btained high yields of penicillin from glucose by feeding the ıgar in solution continuously at the rate of 0.03% per hour. The H was controlled in the optimum range 7.0 to 7.4 by automatic ddition of gaseous ammonia.

The superiority of lactose is presumably due to its slow availbility. No acids accumulate during its utilization, probably beuse any acids formed are more readily available than lactose and e utilized as rapidly as they appear. When carbon sources other an lactose are used, the concentration of the various medium constituents must be such that the pH changes during the fermentation favor good growth and penicillin production. In a corn-steep liquor medium, much of the carbon metabolized i supplied by the corn-steep rather than by the added carbohydrate

In a typical medium, containing as sources of carbon, glucose, corn-steep liquor compounds, lactic acid, and lactose, the mold uses first the glucose and simultaneously some of the corn-steep carbon. When the glucose is completely utilized, the lacti acid is metabolized, together with more of the corn-steep compounds. Lactose utilization begins when other carbon source are exhausted. The period of lactose utilization is the main phas of the penicillin-producing cycle. The utilization of the carbohydrate sources in the described manner tends to produce characteristic pH curve which will be discussed later.

When the lactose is exhausted, no additional available nutrients remain. If the fermentation is allowed to continue, autolysi begins and the penicillin concentration decreases. In commercia operations, the fermentations are harvested before this point because of the difficulties encountered in filtering autolyzed culture and in order to avoid losses of penicillin.

Nitrogen Utilization

Organic nitrogen, in the form of amino acids, peptones, an proteins and inorganic nitrogen, as ammonia and nitrate, can b readily utilized for growth and penicillin production by *P. chrysogenum*. Ammonia is most readily assimilated, as is indicated by th initial drop in the ammonia-nitrogen level of a fermenting cultur A significant portion of the nitrogen assimilated by the mold is i the form of ammonia nitrogen, formed during the fermentation b the deamination of nitrogenous corn-steep liquor compound Usually, an initial increase in ammonia-nitrogen occurs in th medium because it is produced at a faster rate than it is utilize This is followed by a very rapid decrease, which continues until th end of the fermentation. Occasionally, a second rise in ammoni nitrogen occurs, as a result of autolysis of the mold at the end of th fermentation cycle. Nitrate nitrogen appears to be utilized onl when organic nitrogen is no longer available.[30] In a high-corn-stee low-nitrate medium, the organic nitrogen serves as the main sourc of nitrogen. If the corn-steep concentration is reduced and th nitrate increased, the nitrate supplies most of the organism's requirements. The nitrogen incorporated in the cells rises rapidly during the early stages of the fermentation and then levels off. A late decrease in mycelial nitrogen may occur if autolysis takes place.

pH Changes

The pH changes of a typical penicillin fermentation follow a more or less characteristic pattern. At the beginning of the fermentation, the pH is between 5 and 6, depending on the amount and kind of corn-steep liquor used in preparing the medium and the neutralizing agents used. During the first several hours, no change in pH occurs. Glucose and corn-steep compounds are being utilized to build mycelium and any ammonia produced is immediately used. Following this period, the lactic acid is assimilated and ammonia begins to accumulate. This results in a marked rise in pH up to 7 to 7.5, primarily because of the utilization of the lactic acid with the resultant freeing of inorganic cations. As the mold begins utilizing the lactose, the accompanying ammonia assimilation may cause a slight pH drop. Often, however, a stable plateau or a slow rise is observed. Toward the end of the fermentation, a secondary rise in pH to 8 or above may occur, when the carbohydrate of the medium is exhausted and autolysis begins. The pH changes during the fermentation will vary considerably with different media. For example, in a glucose fermentation, as described by Johnson,[28] the pH does not rise until the glucose is exhausted. The pH then rises sharply, followed by autolysis because of lack of nutrients. There is no pH plateau similar to that occurring in a lactose medium. In general, pH values below 7 or much above 8, during the penicillin-synthesis phase of the fermentation, are not associated with good penicillin yields.

Mycelial Development

The development of the mycelium occurs primarily during the first 20 to 30 hours of the fermentation, coinciding with the period of rapid glucose and corn-steep carbon utilization. This growth can be followed by mycelial-nitrogen determinations. In a typical fermentation, the growth is quite appreciable and considerable thickening of the culture takes place. In some cases, a 24-hour culture is so heavy that a sample placed in a 25 mm diameter te tube will remain in the tube when it is inverted. The growth m be in the form of indefinite clumps of mycelium or definite pelle ranging from 0.5 to 2 mm in diameter. During the main penicilli producing phase of the fermentation, little or no mycelial grow takes place. When the nutrients have become exhausted, autoly of the mycelium begins and its weight decreases.

Pigment Formation

The older strains of the penicillin-producing molds usua synthesize, together with penicillin, a number of yellow pigme which impart a bright-yellow color to the fermenting culture. Wh these pigments are usually associated with good yields of penicill their presence in the culture is not always indicative of this c dition. Research by Churchill and Roegner,[12] working in the l oratory of Backus and Stauffer on irradiation of *P. chrysogenum* ultraviolet rays, has resulted in strains which are capable of go penicillin production, but which produce no appreciable amount pigment. Recently workers from the University of Wisconsin h reported on the genealogy and biochemical performance of races of pigmentless mutants obtained from *P. chrysogenum* Q by treatment with ultraviolet light and nitrogen mustard.[3a] N pigment-secreting strains are in general use in indust operations.[48]

Penicillin Formation

The production of penicillin begins as soon as a good amo of mycelial growth has taken place. However, during the first to 30 hours, when growth is rapid, the rate of antibiotic product is comparatively low. The maximum rate of penicillin synth occurs during the main phase of the fermentation, while the lact is being utilized. Throughout this period, the rate of product is rather uniform and the yield curve rises sharply. When medium nutrients are exhausted, penicillin production levels and, if the fermentation is allowed to continue, the penicillin c centration in the medium will begin to decrease. The yield penicillin produced in commercial fermentations is unknown these figures are considered confidential by the various ma facturers. However, yields of 1,500 units per ml have been orted in pilot-plant fermentations[37] and it is generally believed hat industrial-fermentation yields exceed this figure.

Oxygen Utilization

Since the penicillin-producing molds are highly aerobic organisms, a large amount of oxygen must be supplied during the fermentation cycle. This is accomplished by blowing a continuous stream of sterile air into the fermenting medium. Koffler, Emerson, Perlman, and Burris[30] reported uptakes of 109 to 134 ml of oxygen per hour per liter of culture with strain NRRL 832 on a lactose medium. On a glucose medium, the oxygen uptake was considerably higher. The oxygen consumed by the mold is utilized in the aerobic oxidation of carbon compounds and results in the development of large quantities of carbon dioxide during the fermentation. The rate of carbon dioxide development varies proportionately with the oxygen supplied. Johnson[28] reported the production of 2.7 volumes of carbon dioxide per minute per 1,000 volumes of medium at an aeration rate of 0.15 volumes per minute per volume of medium and 10.0 volumes of carbon dioxide at an aeration level of 1.5 volumes. The rate of sugar fermentation obviously depends on the available oxygen and, as is indicated by the carbon dioxide development, varies with the oxygen supply.

Precursors

The types of penicillin produced in the fermentation can be influenced considerably by the presence of certain precursor compounds in the medium. When phenylacetic acid compounds are present,[36] the mold utilizes the phenylacetic residue directly,[7] resulting in a greatly increased production of penicillin G and a decreased production of other types. Other compounds of the proper structure will act as precursors for penicillin X, penicillin F, penicillin K, and dihydropenicillin F. It has also been found possible to produce "unnatural" penicillins, such as *p*-nitrobenzylpenicillin,[21] *p*-iodobenzylpenicillin, *p*-tolylmercaptomethylpenicillin, phenylmercaptomethylpenicillin,[2] *p*-bromophenylmercaptomethylpenicillin, and many others, by supplying the mold with the proper precursor. The effect of precursors on the total yield of penicillin G and on the relative amounts of the respective penicillins is described by Singh and Johnson.[44] A summary of the work on precursors for penicillin is presented in the monograph, *Th Chemistry of Penicillin.*[14]

It should be pointed out that very little information is avai able on the course of the fermentations in industrial practice and is very probable that the characteristics of the fermentations various producers differ considerably. Sufficient data on small-sca operations are available to indicate that minor changes in such fa tors as medium, aeration rate, type of inoculum, etc., will resu in significant alteration of the chemical changes during the ferme tation. The discussion presented here can only be based on da that are available and, at best, can indicate the fundamental cha acteristics of the fermentation. However, it is felt that the publishe work does cover the basic mechanism of the penicillin fermentati and that, although it is certain that various producers have i proved processes, these improvements are the result of variatio which do not involve changes in the fundamental aspects of t fermentation.

COMMERCIAL PRODUCTION OF PENICILLIN

Different procedures for the cultivation of the penicilli producing molds have been studied in considerable detail a several technics developed which involve the surface growth of t mold on a solid menstruum. The use of wood shavings loose packed in a column was one of the procedures that received so attention. In this technic, the nutrient medium was trickled slo through a column of wood shavings on which the mold was growi in a manner similar to that used for the production of acetic ac Another method developed was the propagation of the mold bran moistened with nutrient solution and contained in a slow revolving drum. This technic was developed by one company t full-scale commercial production unit. However, apart from t surface-culture procedure on a liquid menstruum and the s merged-culture process, no other method of production ever achie any economic significance and this discussion will be limited to t two methods which have produced large quantities of penicillin a were of real economic importance.

The Surface-Culture Fermentation

Although the surface-culture method for the commercial p duction of penicillin is no longer used in the United States or England, a brief description of this process will be given, since it was responsible for the first commercial production of penicillin and rather large production units were operated successfully in this manner. Despite the fact that the surface-culture process was expensive, laborious, and commercially and economically impractical, it served to bridge the period between the initial interest in penicillin and the development of the submerged-culture process and made penicillin commercially available when it was desperately needed.

It was natural that the first attempt to produce penicillin commercially should have been by the surface process, since this method is based on the natural tendency of molds to grow as a thin layer on the surface of liquid media. Many variations of the process, with respect to the type of container in which the mold was grown, were developed by different laboratories. The fundamental requirement for the cultivation of the mold under surface conditions is that the depth of the medium should be approximately 2 cm. Any container which will provide a shallow layer of medium is satisfactory. Production units were built from various types of bottles, flasks, and metal or glass trays and pans. Many novel and unusual methods were used for sterilization of the containers and inoculation of the media. Basically, however, all the various production procedures were similar. Mass spore cultures of a suitable strain of *Penicillium* (NRRL 1249-B21, NRRL 1951-B25 and others) were produced on large agar slants in bottles, or on moistened bran or other grain products, such as oat hulls, cracked corn, etc. If desired, an aqueous suspension of the spores was prepared by adding sterile water to spore culture and vigorously agitating to break up the clumps of spores and suspend them in the water. The sterile medium in a suitable container was inoculated by spraying the aqueous suspension of spores into the container or by simply adding a few milliliters of the suspension to the container from a pipette. When dry spores on a grain carrier were employed for inoculum, the material was blown into the container or a few grams merely poured into it. The dry spores gave somewhat more satisfactory results since many spores floated on the surface of the medium, which resulted in more rapid development of the mat. After inoculation, the medium was incubated at 24° to 28°C for 6 or 7 days. Usually, the maximum production of penicillin was obtained in this period and the cultures were then harvested. The containers were emptied and th broth collected, after separation of the mold. In some plants, th medium was withdrawn from the container, without greatly di turbing the mold mat, and replaced with fresh medium. Maximur yields were obtained in the second lot of medium several days earlie than in the original fermentation. This procedure could be r peated several times. The fermented medium was then worked u by procedures similar to those that will be described later in the di cussion on the recovery of penicillin.

The basic principles of the surface-culture process are well i lustrated in the following description of a laboratory experimen

Sixty-five milliliters of a medium containing, per liter, 50 corn-steep solids, 30 g technical lactose, 3 g $NaNO_3$, 0.5 g KH_2PO 0.25 g $MgSO_4$, 0.2 g $C_6H_5CH_2COOH$, 0.044 g $ZnSO_4$, and NaO to pH 5.5, were placed in a 300-ml Erlenmeyer flask. The flask w stoppered with a cotton plug and sterilized 30 minutes in an aut clave at 15 psi steam pressure. After cooling, the flask was inoc lated with one loopful of dry spores from a 6-day agar-slant cultu of *P. notatum* NRRL 1249-B21. The flask was incubated at 24° and samples were removed for assay after 5, 6, 7, and 8 days. Th following results were obtained:

	5 days	*6 days*	*7 days*	*8 da*
Penicillin, units per ml	65	142	193	136
pH	7.2	7.6	8.1	8

The difficulties encountered in the operation of industri production units, using the surface-culture process, were tremendo Any significant volume of production required a great many co tainers, huge incubators, a large labor force, and a relatively lar investment in buildings and supporting equipment. Some plar handled 50,000 or more bottles daily. Even plants using trays pans had to process thousands of them a day to achieve any signi cant production and, despite the enormous amount of materi space, and labor that were used in the surface-culture plant, t daily production of fermented medium amounted to only a fe thousand gallons. The sterility problems involved in the surfa culture alone were great enough to make this method unfeasib for economical large-scale production. Because of the great di culties encountered and the inherent disadvantage of the proce as soon as the submerged-culture process was developed to the po of large-scale operation, the surface-culture method was abandoned by all penicillin producers.

The Submerged-Culture Fermentation

Although the submerged-culture process for the commercial production of penicillin is fundamentally simple, the actual production unit is a very intricate and complex mechanical system. The modern penicillin plant is a very highly engineered unit, which depends for successful operation on the perfect integration and control of a large amount of machinery and is essentially a mechanical process requiring human effort only to coordinate the functioning of the machinery and to keep it running smoothly. The first impression one gets on visiting a penicillin plant is that there seem to be very few people required for the operation of a relatively great amount of mechanical equipment. From an engineering standpoint, the development of the process required a tremendous amount of engineering research, including the design of special unique equipment as well as the adaptation of much standard machinery to rather unusual uses. A relatively modest submerged-culture plant requires an investment of several million dollars in building and equipment and the services of highly skilled personnel to supervise its construction and operation.

The development of the submerged-culture process, made possible by the discovery of strains of *Penicillium* suitable to this type of operation, was the greatest single factor responsible for the truly large-scale production of penicillin, which has made possible the manufacture of tremendous quantities of this remarkable chemotherapeutic drug at an extremely low cost. Although this development took place within a relatively short period of time, in terms of research effort, tremendous investment was required. More than twenty laboratories, both governmental and private, were concerned with this work and contributed to its successful completion. Even yet, much continuous study is being devoted to the submerged-culture process and improvements in yields are being achieved constantly.

Fundamentally, the submerged-culture process is as simple as the surface method, consisting essentially of growing a suitable strain of *Penicillium* homogenously dispersed throughout a volume of liquid medium. The process is illustrated by the following description of a shake-flask experiment: One hundred and fifty mil liters of medium, containing, per liter, 12.5 g corn-steep solic 20.0 g lactose, 5.0 g Cerelose, 3.0 g $NaNO_3$, 2.0 g KH_2PO_4, 0.04 $ZnSO_4$, 1.0 g $CaCO_3$, and 0.5 g phenylacetic acid derivative, co tained in a 1 qt Mason fruit jar, Kerr type, was sterilized minutes in an autoclave at 15 psi steam pressure. The flask w then inoculated with 6 ml of a 48-hour vegetative culture of *P. chr ogenum* Q176. (The inoculum culture was prepared by inoc lating a flask of the described medium with 1 ml of an aqueo spore suspension made by suspending the spores from a 6-day aga slant test-tube culture in 5 ml of sterile water; the flask was th incubated 48 hours at 24°C in the shaking machine.) The inoc lated flask was incubated at 24°C in a rotary shaker having a 2. in. diameter movement and rotating at 180 rpm. Samples we taken at 3, 4, 5, 6, and 7 days for penicillin assay. The results we as follows:

	4 days	*5 days*	*6 days*	*7 da*
Penicillin, units per ml	492	602	545	560
pH	7.4	8.1	8.6	8

Although each penicillin manufacturer developed his ov method and the details of the various producers' processes are n available for comparison, it is generally thought that these process are essentially similar and the differences that do undoubtedly ex are ones of minor technical nature. In general, the submerge culture fermentation process consists of three steps: (1) Preparati of inoculum, (2) production fermentation, and (3) harvesting t fermented medium.

In this review, it will be impossible to describe in detail the various types of production units in operation because of t limited information available. Each manufacturer's plant diffe in some degree from all others, because each was developed more less independently. However, since the various plants are func mentally the same, it will suffice for our purpose to describe wh may be considered a typical production unit, one which will ill trate the basic design of a submerged-culture penicillin-producti plant. Figure 10 is a diagrammatic flow sheet of this typical u and shows the various steps in the process. Recently, another fl sheet and brief description of the process has been publishe

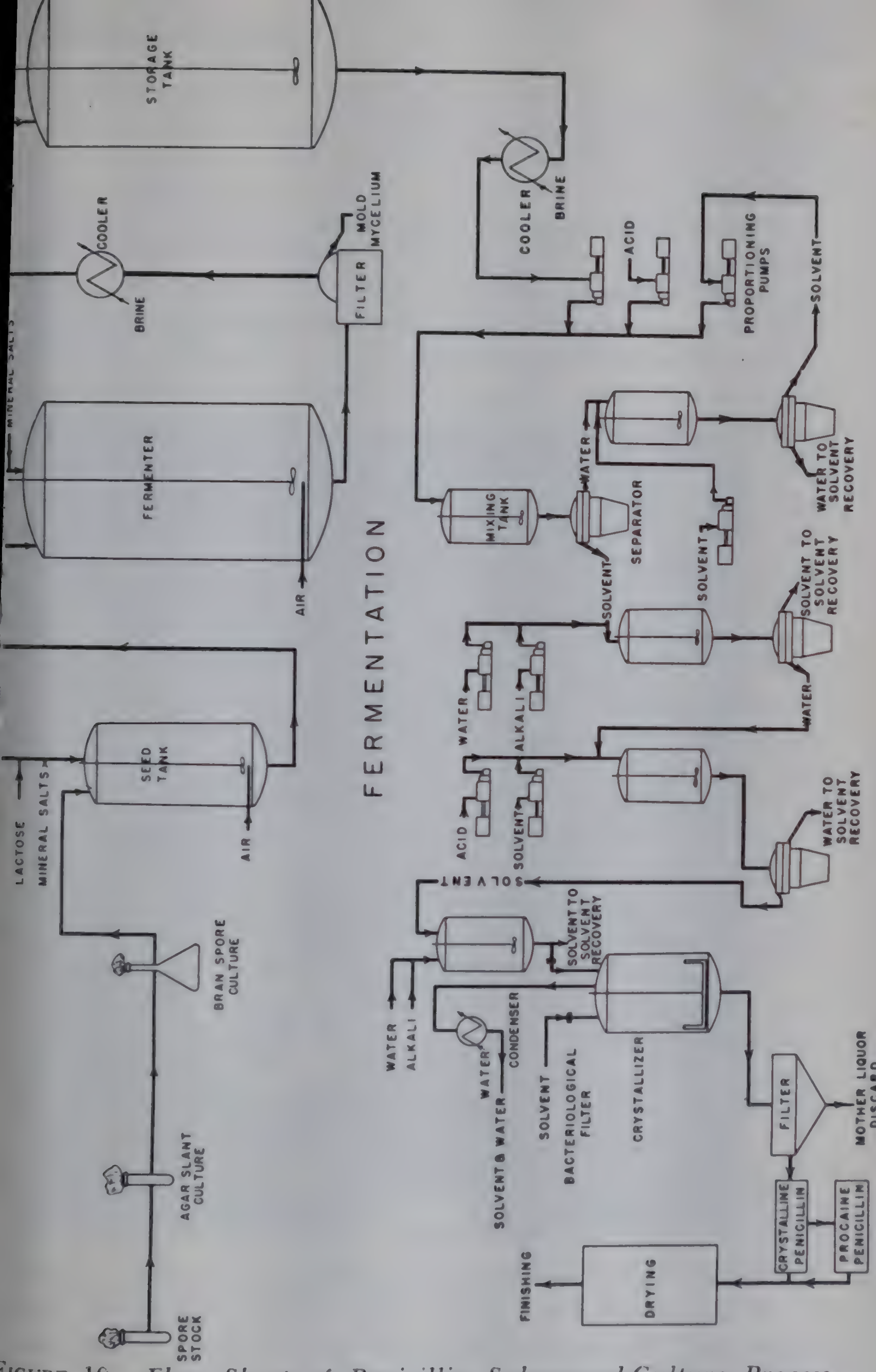

STORAGE TANK
COOLER
BRINE
FILTER
MOLD MYCELIUM
MINERAL SALTS
FERMENTER
AIR
SEED TANK
LACTOSE
MINERAL SALTS
AIR
BRAN SPORE CULTURE
AGAR SLANT CULTURE
SPORE STOCK
FERMENTATION
COOLER
BRINE
ACID
PROPORTIONING PUMPS
SOLVENT
MIXING TANK
SEPARATOR
WATER
SOLVENT
WATER TO SOLVENT RECOVERY
SOLVENT
SOLVENT TO SOLVENT RECOVERY
WATER
ACID
ALKALI
WATER
ACID
SOLVENT
WATER TO SOLVENT RECOVERY
SOLVENT
SOLVENT TO SOLVENT RECOVERY
WATER
ALKALI
CONDENSER
WATER
SOLVENT & WATER
SOLVENT
BACTERIOLOGICAL FILTER
CRYSTALLIZER
FILTER
MOTHER LIQUOR DISCARD
CRYSTALLINE PENICILLIN
PROCAINE PENICILLIN
DRYING
FINISHING

FIGURE 10. *Flow Sheet of Penicillin Submerged-Culture Process*

The various phases of the process will be considered in the that they function in the commercial production unit.

PLANT EQUIPMENT

The equipment for growing the penicillin-producing n consists essentially of various-sized fermentation tanks whic used for two purposes: to grow mold for inoculum and to mold for penicillin production. The tanks used to grow inoc are referred to as seed tanks, while those in which the penicil produced are called fermentors. Usually, the seed tanks fermentors are of identical design, with the exception of ce minor modifications required for their particular function. tanks may be constructed of carbon steel, stainless steel, In clad steel, or plastic-coated steel. The last three materials certain advantages over plain carbon steel from the standpo cleaning and corrosion and may be slightly superior fron standpoint of yield. The tanks are equipped with mech agitators, a sparging system for aeration, and usually with connected to high-pressure steam for sterilization and to wat cooling. Where the medium is sterilized before being put int tank, the cooling system may consist of a jacket around the the coil being eliminated.

Many different types of agitators are in use. They are u of a turbine type to impart a definite rotary motion to the l Baffling may or may not be used, depending on the type of ag employed. High-speed agitation which will finely disperse t in the liquid is required, necessitating relatively large moto heavy drives. The tanks are cylindrical and their height is u two to three times the diameter. The seed tanks commonly capacity of about 10% of the fermentor. Table 32 indicates general way, some of the characteristics of the fermentation

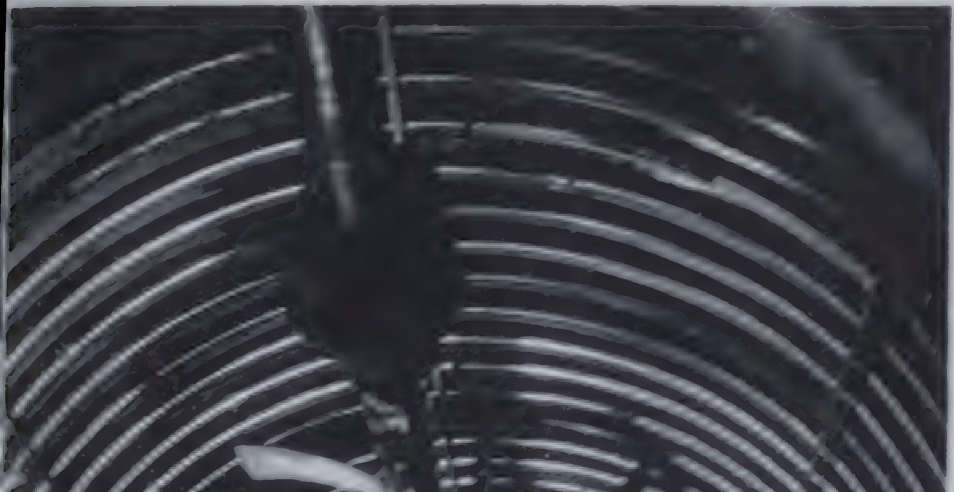
TABLE 32. FERMENTOR CHARACTERISTICS

Type of tank	Total capacity gal	Working capacity gal	Agitator rpm
Seed	500	350	240
Fermentor	5,000	4,000	120

As aeration causes a great deal of foaming during the of the mold, the tanks are equipped with automatic devices add defoamer whenever the foam reaches a certain level.

Various means are used to distribute the air that is intr

tanks. Usually, the air outlets are immediately below the
rs, as this arrangement provides better dispersion of the air
r outlet may consist of a pipe with small holes along the
d arranged in the form of a square or circle, of jets, shower
r, even of a single pipe open at the end. Carborundum
r other air-dispersing materials of extremely fine porosity
suitable since the mold would grow in the small openings,
plugging during the run. Figure 11 shows the interior of a
al fermentor.



11. *Interior of a 5,000-gal Fermentation Tank* (Courtesy
–Cutter Laboratories)

he seed tanks and fermentors are connected by piping in such
that they form an entirely closed system, adequately pro-

tected from the atmosphere and capable of operation withou
posing the medium to contamination. The inoculum is trans
from the seed tank to the fermentor by air pressure. During o
tion, a 5 to 10 psi air pressure is maintained on the tanks.
not in use, all piping connecting the seed tank and fermentor
all other connections to the tanks, are maintained under 15
psi steam pressure. It is absolutely essential in the operation o
fermentation tanks that the system is kept free of contamina

Large quantities of compressed air are required for the gr
of the mold and, since this air must pass through a system of
siderable resistance, supply pressures in the range of 20 to 5
are required. Air compressors capable of delivering several thou
cubic feet of air per minute at these high pressures are empl
Centrifugal blowers can be used if a multi-stage system, ca
of building up the desired pressure, is employed.

Several different methods are used to sterilize the air. O
the most common is by filtration through carbon or glass
A filter using these materials consists of a large pipe which is pa
with the filtering agent and provided with a steam inlet for ster
tion. The filter is sterilized under 15 to 30 psi steam pressur
rectly on the filtration agent. A typical filter capable of steril
up to 500 cfm of air is constructed of 18 in. steel pipe, 10
length with flanged ends. It contains a column of carbon (10
mesh) supported on a fine wire mesh and will operate satisfac
for many months without changing the carbon. This filt
sterilized each time a run is made in the tank. Other metho
air sterilization employ scrubbing towers, containing germi
solutions, and incineration. One plant apparently obtains
factory operation by placing the air compressors close to the
and depending on the heat generated during the compression
air for sterilization.

PREPARATION OF INOCULUM

Since the *Penicillium* strains used for the manufacture of
cillin are subject to extreme variation, the handling of the cu
and the preparation of inoculum for the production ferme
must be carefully controlled. A master stock of the orga
P. chrysogenum, in the form of soil stocks, lyophilized stock
frozen slants serves as the starting point for each fermentor
Inoculation of a production fermentor from another produ

mentor is not used except as an emergency measure if the
oculum prepared for that particular run cannot be used. The
t step in the preparation of inoculum, in a typical process, con-
ts of transferring a few spores from the master stock to a test-tube
ar slant of a medium that will provide optimum sporulation.
e sporulation medium, developed by Moyer at the Northern
egional Research Laboratory, gives excellent sporulation. This
edium contains, per liter, 12.5 g molasses, 7.5 g glycerol, 5.0 g pep-
ne, 4.0 g NaCl, 0.25 g $CaCO_3$, 0.05 g $MgSO_4$, 0.06 g KH_2PO_4,
003 g $FeSO_4$, 0.001 g $CuSO_4$, and 15.0 g agar. The agar-slant cul-
re is incubated at 24° to 26°C until good sporulation occurs,
ually 5 to 7 days. The spores from the tube are then suspended
sterile water and used to inoculate a bottle or flask containing
eat bran moistened with a nutrient solution. The formula for
e bran medium contains 667 g wheat bran moistened with 333 ml
trient solution which has the same composition as the sporulation
edium except that it contains no agar and the concentration of
l ingredients is increased fourfold.

The bran cultures are incubated at 24°C until heavy spore
rmation has taken place (5 to 7 days). These spores are used to
oculate the seed tank containing liquid medium. Various media
ay be used in the seed tanks and the exact composition must be
etermined experimentally. However, satisfactory results may be
btained employing media similar to that used in the production
rmentor. The medium is sterilized prior to inoculation and the
ansfer of the spores to the tank is made with careful aseptic
ecautions. After inoculation, the tanks are held at a constant
mperature and continuously agitated and aerated. The tempera-
re control is achieved through the use of automatic control de-
ces. This factor is critical. The number of spores used for
oculation, the medium, and the incubation temperature vary,
epending on the time cycle desired and the characteristics of the
ed for the particular plant. These factors have to be determined
perimentally on the production equipment. Usually, the time
cle is relatively short, ranging from 24 to 48 hours. Very heavy
ycelial growth is obtained in this period under the proper con-
tions. Seed tanks for growing inoculum are illustrated in
gure 12.

An alternate method of preparing inoculum consists of de-
loping sufficient vegetative mycelium in small containers for

inoculating the seed tanks. This can be done, for example,
inoculating shake flasks, containing 100 to 150 ml of liquid medi
with spores from an agar slant and incubating the flasks for 4
72 hours on a shaking machine at 22° to 26°C. The vegeta
growths from several flasks are then combined and used to inocu
the seed tank. Under good conditions, about 1,000 ml of vegeta
mycelium will suffice for 200 to 500 gal of seed medium.

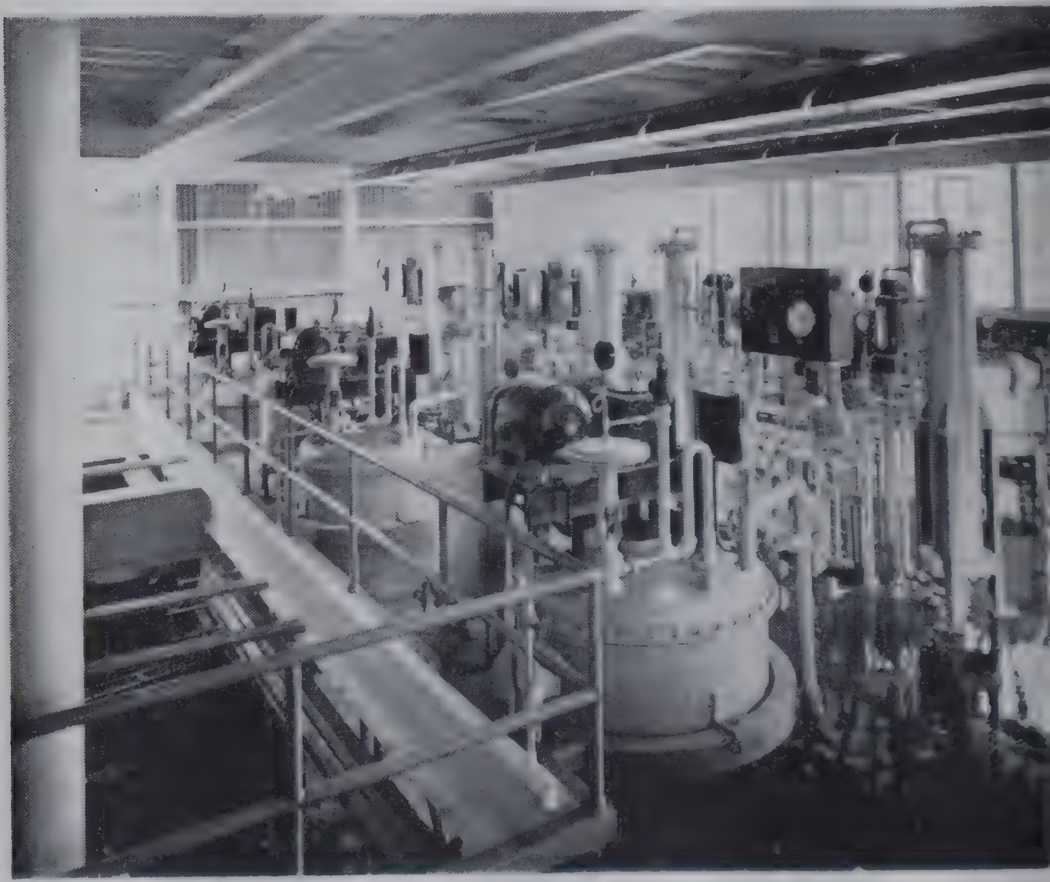


FIGURE 12. *Seed Tanks for Growing Inoculum* (Courtesy—Al
Laboratories)

If the production fermentors are large, in the range of 2
gal, it may be necessary to build up the inoculum through two
instead of one. In this case, the first seed tank is used to inoc
the second larger tank, which then is used to inoculate the
duction fermentor.

During the development of inoculum in the seed tanks, sar
are withdrawn periodically and examined for growth and
tamination. Both microscopic examination and subculture
broth medium are made to check on possible contamination

cteria or yeasts. Only when it is certain that the seed culture has own satisfactorily and is free of contamination is it used.

ODUCTION FERMENTATION

The production fermentation is carried out in tanks of 2,500 20,000 gal capacity. The tanks are filled to about 75% of pacity with the medium, which may be sterilized in the fermentor continuously as it is fed into a presterilized tank. A tank con-ining 4,000 gal of medium requires approximately a 4-hour erilization cycle when 100 psi steam pressure is used in the coil. pproximately 90 minutes are required to bring the temperature of e medium to 120°C, where it is held for 30 minutes. An additional 0 minutes are needed to cool the medium sufficiently to permit oculation. Sterilization with batch cookers, followed by cooling th tubular heat exchangers, is also employed. The exact compo-tion of the medium used may vary, depending on a number of ctors, and each producer has his own formula. Fundamentally, wever, most production media are similar. A typical medium ntains, per liter, 30.0 g corn-steep solids, 30.0 g lactose, 5.0 g ucose, 3.0 g $NaNO_3$, 0.25 g $MgSO_4$, 0.044 g $ZnSO_4$, 0.05 g phenyl-etamide, and 3.0 g $CaCO_3$.

GURE 13. *Production Fermentation Tanks* (Courtesy—Abbott Laboratories)

The various ingredients are mixed in a small tank with s cient water to make an easily pumpable solution. The charg then pumped into the fermentor and water added to the des volume. The use of a medium-mixing tank has many advant over mixing in the fermentation tank and is often used. If a tinuous sterilization process is used, the medium is prepared in final form in a reservoir tank and pumped through a sterilizer the fermentor. Typical production fermentors are shown Figure 13.

When the sterilized medium is ready for inoculation, the tents of the seed tank are blown into the fermentor. The inocul medium is then continuously agitated and aerated for a perio several days. The temperature is maintained constant in the ra of 22° to 28°C by the use of automatic controls. The length of fermentation cycle varies from 40 to 96 hours in different pla depending on the manner in which the plant is operated. various factors affecting the fermentation are so controlled that maximum yield will be obtained by the time specified for har Most plants are operated on a definite schedule which does not mit much variation in the over-all fermentor cycle. Samples

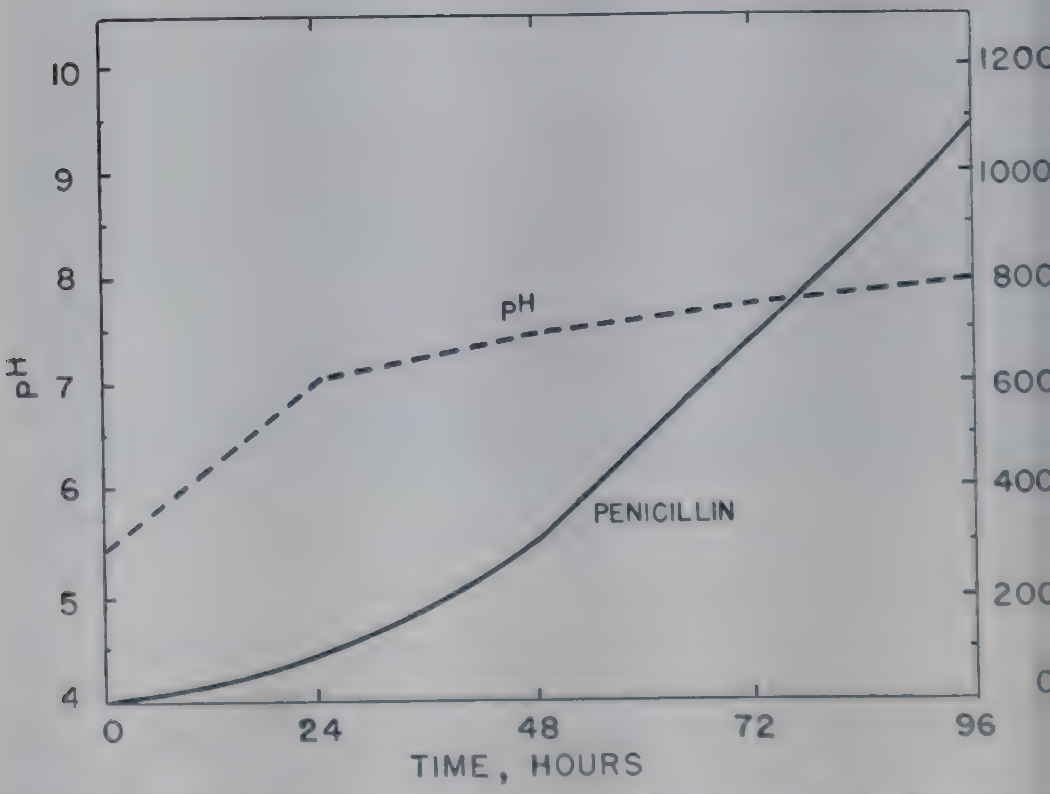
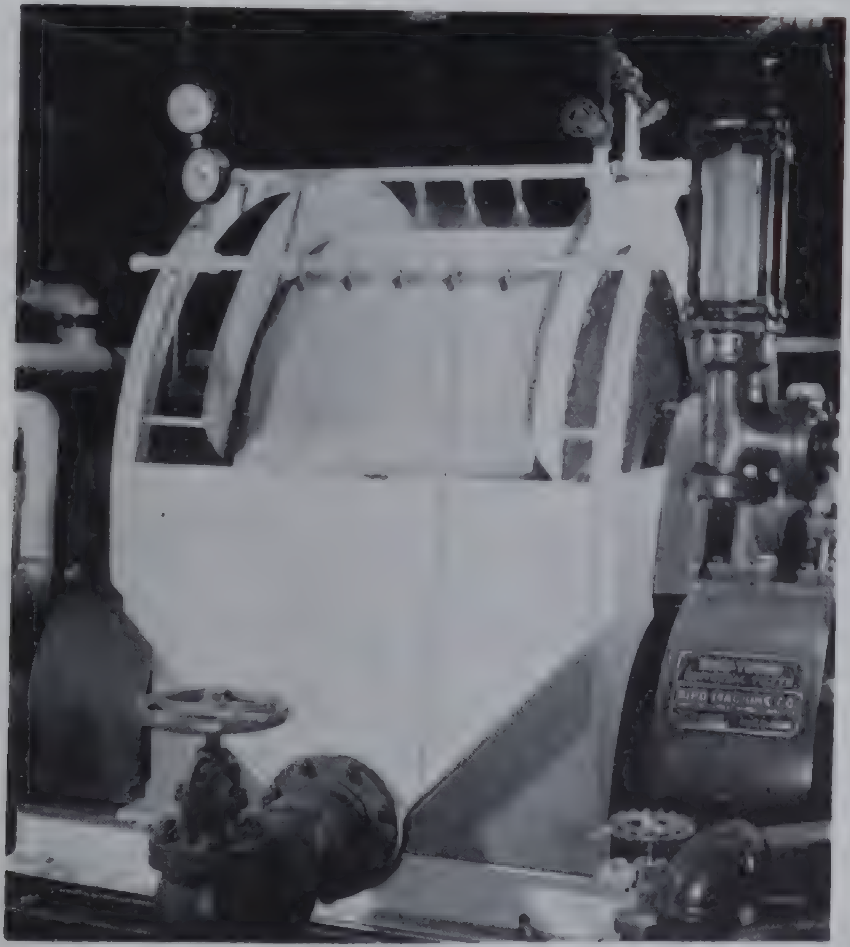


FIGURE 14. *Fermentation Cycle in a 5,000-gal Fermento*

moved from the fermentor at periodic intervals for penicillin say and examination for contamination. A typical fermentation cle in a 5,000 gal fermentor is shown in Figure 14.

ARVESTING THE FERMENTED MEDIUM

At the time of harvest, the fermented culture is light tan and about the consistency of light sludge. The culture is blown or umped into a reservoir tank which feeds a continuous, rotary cuum filter. The mycelium is separated from the liquor, washed the filter, and discharged as a thick blanket, resembling paper ulp. The penicillin-containing beer is pumped from the filter, rough a heat exchanger which cools it at 2° to 4°C, into a storage nk. As the filtration step is not a sterile operation, it is desirable chill the beer as soon as possible after it leaves the filter. Between ns, the filter is usually sterilized with steam or germicidal solution. is essential that growth of bacteria is not allowed to build up in



GURE 15. *Rotary Vacuum Filter for Separating the Mycelium from the Broth* (Courtesy—Abbott Laboratories)

the filtration system, because this can result in severe destruc of penicillin by the action of bacterial penicillinases. The capa of the rotary filter varies with the size of the drum, ranging upw from 2,000 to 3,000 gph for a drum 4 ft in diameter and 28 in. w A typical rotary vacuum filter is shown in Figure 15.

To produce 1,000 gal of fermented culture (5 to 6 pound penicillin) by the submerged-culture process requires approxima 500 lb of nutrients, 7,500 lb of steam, 10,000 gal of water, 1,000 of electricity, and 250,000 cu ft of air.

Recovery of Penicillin

Two processes for the recovery of penicillin have been use commercial production. One process is based on the adsorptio penicillin from the beer on carbon, while the other is based on extraction of penicillin from the beer at an acid pH with wa immiscible organic solvents. The carbon process was widely during the early period of penicillin production, but has lar been supplanted by the solvent process. The primary advantag the solvent process over the carbon process is that for the sec method, increased amounts of carbon are required as the con tration of penicillin in the beer increases. In contrast, incre volume of solvents is not generally necessary with higher-pot beers. Also, there are several handling and operating disadvant inherent to the use of carbon on a large scale in the productio penicillin.

THE CARBON RECOVERY PROCESS

The filtered beer is pumped into an agitated tank, the de amount of activated carbon added, and the slurry mixed fo to 30 minutes. The exact amount of carbon used depends on concentration of penicillin in the beer and will range from 2 to by weight. One gram of carbon will adsorb approximately 2 units of penicillin. The type of carbon used is determined on basis of its relative bulk, wetting ability, penicillin adsorption elution, and filtration characteristics. Such carbons as Nu C-190-N and Darco G-60 give very satisfactory results. No pH justment is made in this step, because the normal pH of the fil beer (pH 7 to 8.5) is satisfactory for good adsorption. When sorption is complete, the carbon is collected by filtration[19] late-and-frame press or by centrifugation in a basket centrifuge. he plate-and-frame press has many advantages over centrifugation. ontinuous, rotary filters may also be employed for this filtration. pproximately 90% of the penicillin is adsorbed under good conditions, leaving about 10% unadsorbed. After washing with water, e carbon adsorbate is eluted. The elution may be accomplished several different ways, the most preferable being with 80% cetone. This eluting solution has a short contact time, high filtration rate, good volume reduction, and high elution efficiency, as ell as other advantages over other eluting agents. The volume of luting solution used varies from 0.05 to 0.5% on the volume of the eer. Either stirred elutions or static elutions may be used. In the rst method, the carbon adsorbate is placed in an agitated tank, ontaining the eluting solution, and stirred for 30 to 60 minutes. The carbon is then filtered off and the eluate collected. In the static lution, the carbon adsorbate is left in the press and the eluting olution pumped through the press. Recycling of the eluting solution may or may not be used, depending on the particular equipment that is employed. In either method, a second elution can be used which is then employed as the first elution of the next batch. While the recovery of penicillin in the eluate will vary, depending on a number of factors, in both methods, 60 to 80% of the penicillin will be eluted in the first elution. The second elution removes an additional 10 to 20% giving an over-all elution of 70 to 90%. The total recovery of penicillin from the beer to this point is usually about 60 to 70%.

The 80% acetone eluate is next concentrated, either by repeated extractions with a water-immiscible solvent[32] or by distillation or evaporation under vacuum[45] at 60° to 90°F. After the acetone is removed, the remaining aqueous solution of penicillin is cooled at 2° to 3°C, acidified at pH 2 to 2.5, and extracted with amyl acetate or a similar solvent in the same manner as will be described in the discussion of the solvent process.

THE SOLVENT RECOVERY PROCESS

The solvent-extraction process is based on the fact that penicillin is preferentially soluble in water or organic solvents depending on whether it is in the form of the free acid or a salt. An idealized distribution curve of penicillin between water and a solvent is shown in Figure 16.

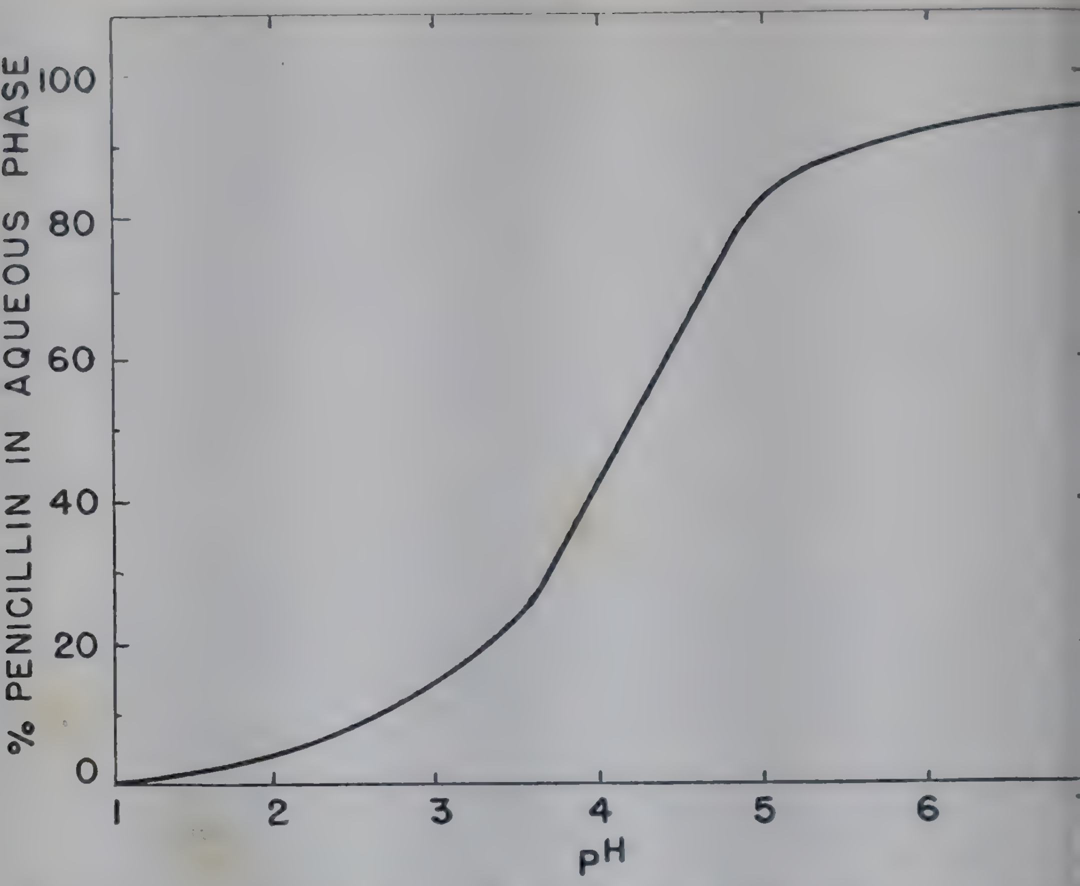


FIGURE 16. *Distribution of Penicillin between Water and Amyl Acetate*

A very good discussion of the theoretical aspects and princip
of the solvent extraction of penicillin has been given by Rowl
Steiner, and Zimkin.[43] Whitmore[47] described a laboratory, c
tinuous, counter-current extraction process.

Several solvents have been used for the extraction of pe
cillin on a large scale, but the most widely used solvents are an
acetate, methyl isobutyl ketone, and butyl acetate. The choice
a suitable solvent depends on a number of factors, such as c
solubility in water, flash point, toxicity for humans, ease
handling, etc. Although many solvents, as for example, met
isobutyl ketone, methylcyclohexanone, and others have grea
solvent power for penicillin than the commonly used solven
these latter appear to be more suitable from an all-around sta
point. Certain specific conditions of the process, such as p
type of deemulsifier, etc., are dependent on the solvent employ
Basically, however, the process is the same, regardless of the solv
used.

The filtered beer, chilled at 2° to 3°C, is first clarified by filtration with 1 to 1.5% Hyflo through a plate-and-frame filter press or a rotary filter. The removal of certain dissolved solids can be effected by adding to the beer, before filtration, aluminum sulfate,[31] tannic acid,[31] or similar substances that precipitate the dissolved solids, which are then removed with the suspended solids in the filtration. This clarification step is not essential, but it reduces the emulsion difficulties encountered in the extraction steps and also lowers the amount of insolubles which collect in the centrifuges. Where the Podbielniak extractor (made by Podbielniak, Inc., 341 E. Ohio Street, Chicago, Ill.) is used, it is very important to clarify the beer. The amount of solids removed by the clarification step varies widely in different beers and while, in certain instances, it is possible to process a beer without clarification, many are encountered which cannot be handled without polishing. Most plants, as a routine, clarify their beers before processing and, although a few plants apparently operate satisfactorily without this step, it is felt that clarification should be adopted as standard practice.

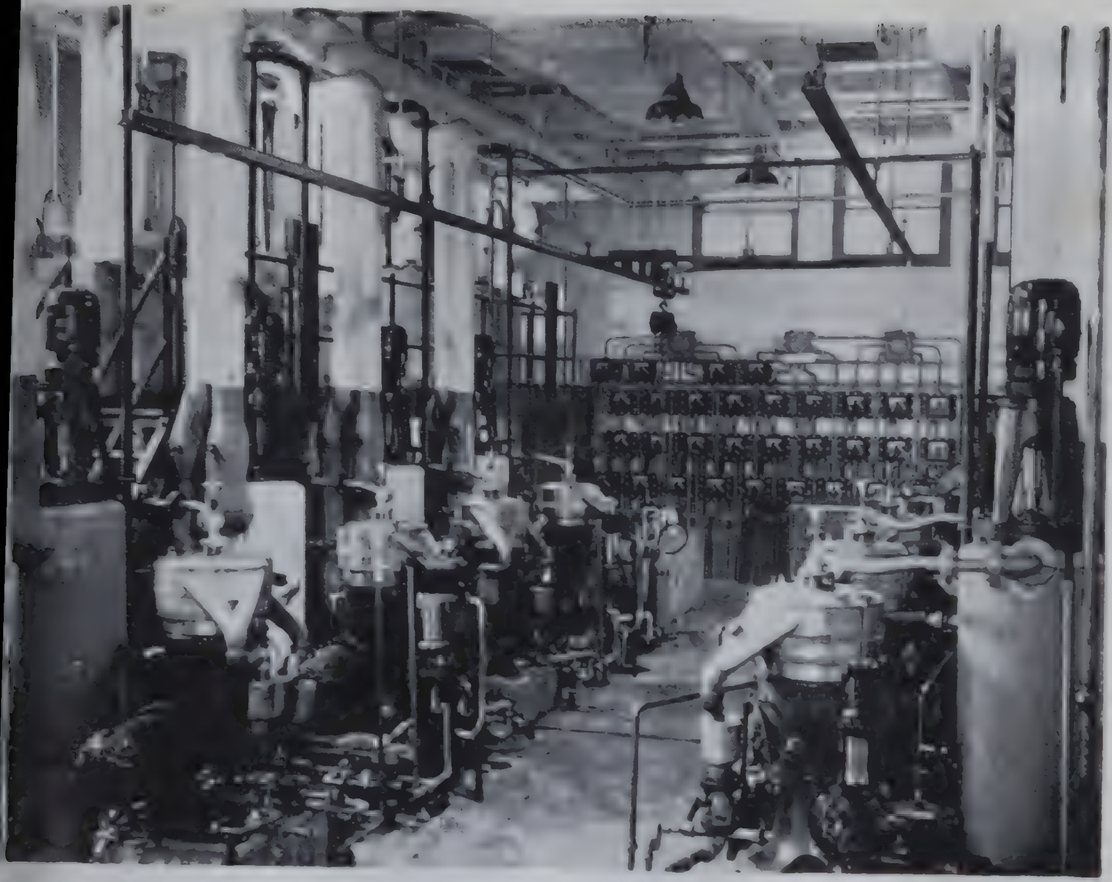
Proportioned flows of broth, acid, and organic solvents are required in the penicillin-recovery process. This is accomplished by the use of proportioning pumps of the positive-displacement piston-type, which accurately deliver any desired liquid flow. The clarified beer, at 2° to 3°C, is fed continuously through the proportioning pumps into the solvent-extraction system. The organic solvent and acid (phosphoric or sulfuric) are similarly proportioned into the system. Usually, the volume of solvent used is approximately 0.2 to 0.4 of the volume of the beer. The acid flow is such that a pH of 2.0 to 2.5 is maintained. A constant-recording pH meter is standard equipment, because the pH must be very carefully controlled. The proportioned flows are fed continuously to a mixing tank where they are intimately mixed and discharged as a fine emulsion. The mixing tank is relatively small (15 to 20 gal) and is equipped with a high-speed agitator. A small mixing tank is necessary because a hold-up time of 60 to 90 seconds in the tank is desired.

The water-organic solvent emulsion is separated into two phases by centrifugation in either disc-type or bowl centrifuges. The through-put capacity is somewhat higher with the disc-type machines. The aqueous phase is discharged from one spout, the solvent phase from the other. The standard-size centrifuges ha a capacity in the range of 1,000 gph of emulsion. It is essent to have a small amount of a good deemulsifying agent (Ultraw 30E or Santomerse S) present in the solutions being processe to facilitate the splitting of the emulsion. The deemulsifying age can be added to the beer before it enters the system, or it can proportioned into the system before the mixing tank. The aqueo phase discharged from the centrifuge is fed into a second mixi tank, along with fresh solvent, and then passed to a second ce trifuge. The aqueous discharge from this machine goes to a s vent-stripping column for solvent recovery and is then neutrali with caustic soda and discharged to the sewer. The solvent fro the second centrifuge passes to a pump feeding the first stage the system and is mixed with the incoming fresh beer. Thus two-stage extraction is accomplished. If desired, a three- or fo stage system can be used. The solvent discharge from the fi centrifuge is pumped to a storage tank preparatory to furth processing.

A battery of centrifugal separators used in the solvent- covery process is shown in Figure 17.

The Podbielniak centrifugal, counter-current solvent extr tor[29] is used in some plants in place of the mixing tank and ce trifuge. This machine is essentially a counter-current extrac with a multiple-stage effect. The main component of the mach is a stainless-steel rotor which revolves at approximately 2,0 rpm. The rotor speed used varies with the solvent. Actually, principle of the Podbielniak extractor is the same as that o counter-current extraction column or a multiple-stage, count current system using mixing tanks and centrifuges. In a syst employing Podbielniak extractors, the chilled, clarified beer, c taining a deemulsifier and acidified to the proper pH, is pump under pressure into the rotor of the extractor. The solvent un pressure is also pumped into the rotor, but at an opposing po to the beer. The two liquids pass rapidly through the rotor opposite directions and are discharged separately. Since the Podb niak extractor is equivalent to several extraction stages, one p sage is sufficient for complete extraction. The aqueous ph passes to the solvent-recovery unit and the solvent phase to storage tank.

A considerable amount of purification is effected in the f



'IGURE 17. *Centrifugal Separators Used in the Solvent-Recovery Process* (Courtesy—Chas. Pfizer and Co.)

xtraction of penicillin. In the clarified beer, the solids have penicillin potency of about 30 units (18 μg) per mg. The solids n the solvent solution have a potency of 200 to 400 units per ng. Under satisfactory operating conditions, 90 to 95% of the enicillin is extracted.

If it is desired, the solvent may be treated with a small amount f carbon (0.25 to 0.5%) to remove certain pigments and other mpurities. The importance of this step depends largely on the eer being processed and may not be necessary.

The next step in the process is to extract the penicillin from he organic solvent into water. The volume of water used is enerally 0.02 to 0.1 of the volume of solvent. The solvent, water, nd sufficient alkali (sodium or potassium hydroxide), or a buffer olution to maintain a pH of 6.5 to 7.0, are proportioned into a ystem identical to that used in the first extraction step, except hat only a single stage is employed. A Podbielniak extractor may lso be used for this step. No deemulsifier is required. After ntimate mixing, the two phases are separated; the solvent passes to the recovery unit and the aqueous phase to the next processi step. A 90 to 95% recovery of penicillin is obtained in this c traction and the potency of the penicillin is increased to appro mately 600 to 800 units per mg. The volume of solution at th point is roughly 0.01 to 0.004 of the original beer volume.

The chilled aqueous solution is next acidified and extract with an organic solvent. This may be done continuously in t same type of equipment as already described or it may be do batchwise in agitated tanks. No emulsion problems are encounter in this step and the two phases separate readily without centrifug tion. Usually a different solvent is employed in this step, becau a greater degree of purification is achieved if a solvent other th the one used in the first extraction step is employed. Chlorofor ether, and other solvents will give satisfactory results.

The penicillin is again extracted into water at pH 6.5 to by titration with a solution of base. The base used here deper on which salt of penicillin is desired as a final product. Co mercially, the sodium, potassium, and small amounts of calciu salts are produced. If the amorphous salts of sodium, potassiu and calcium are desired, the solution obtained at this point freeze-dried and finished without further processing. Most of penicillin, however, is processed to the crystalline sodium or pot sium salt.

Several widely different procedures[1,14,18,19,22,32,38] are descri for producing crystalline salts of penicillin and it is doubtful t any two commercial producers employ identical methods. C method, which apparently gives very satisfactory results, cons of filtering an aqueous solution of sodium or potassium penici (100,000 to 200,000 units per ml) through a bacteriological fi and placing it in a crystallizer. A water-immiscible solvent, s as butanol, also filtered, is added to the crystallizer and an a tropic distillation under vacuum is carried out. As the last the water is removed, the penicillin salt crystallizes and is t collected aseptically on a filter. The crystallizing operation m be carried out under aseptic conditions to avoid contaminat of the product with microorganisms. The crystalline salt is t removed from the filter and dried *in vacuo* at 200 to 400 micr pressure. The dried salt is screened to size and then finished i the various pharmaceutical forms (Figure 18).

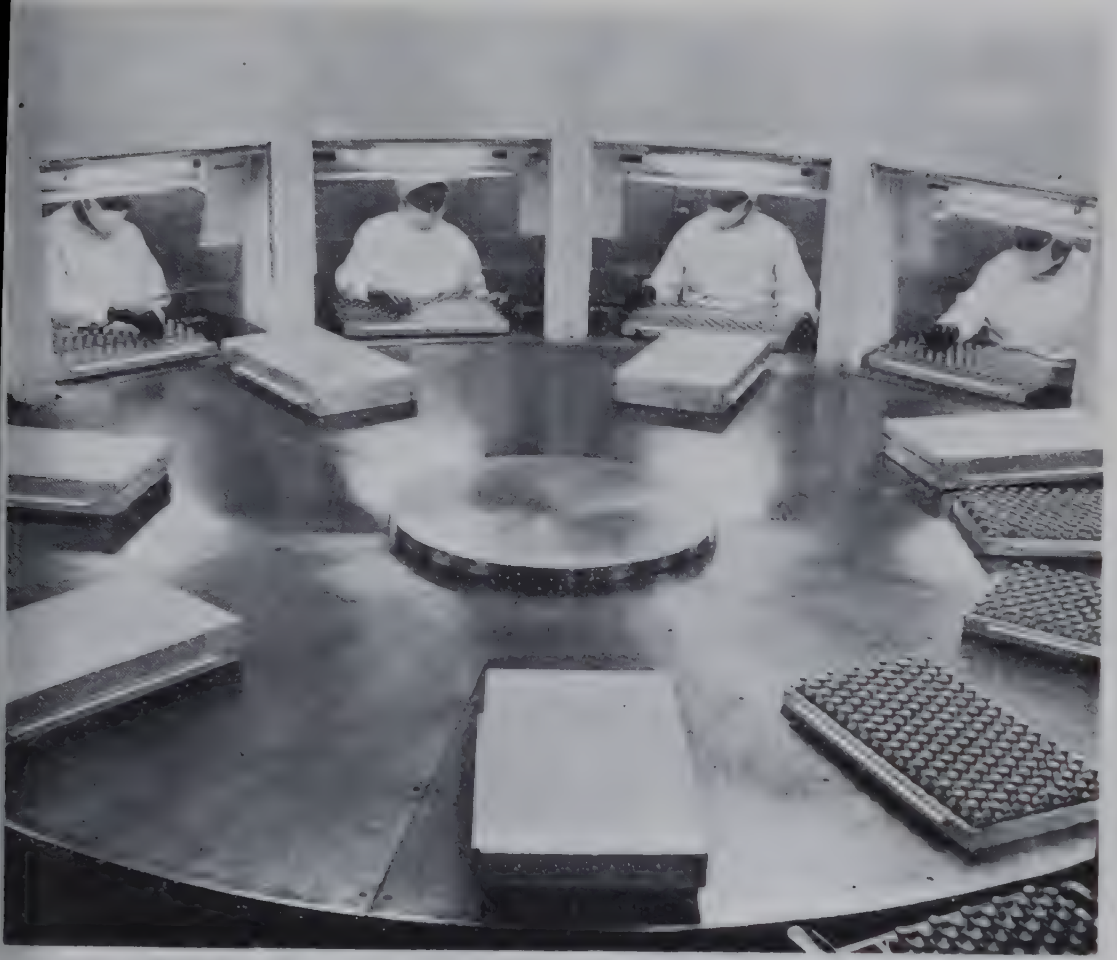


FIGURE 18. *Stainless-Steel Turntable Used in Finishing Operations* (Courtesy—Chas. Pfizer and Co.)

Procaine penicillin may be made from either the potassium or sodium salt by dissolving the crystals in water and adding procaine hydrochloride. Procaine penicillin crystallizes from the aqueous solution and is filtered off and dried *in vacuo*. An alternate procedure that may be used consists of preparing a solution of penicillin (free acid) in an organic solvent and adding a soluion of the free procaine base. The procaine penicillin salt crystalizes readily from the solvent solution.

Penicillin Standards

After manufacture, all penicillin salts must be carefully tested or quality and conformity to the standards set up by the various governments. In the United States, sodium or potassium penicillin G conforms to the following standards, all tests being performed by the methods stipulated by the USP: (1) It is sterile; (2) it is nontoxic; (3) it is nonpyrogenic; (4) it contains not less th 85% by weight of the sodium or potassium salt of penicillin (5) its moisture content does not exceed 1.5%; (6) its pH aqueous solution, at a concentration of 30 mg per ml, is not l than 5.0, nor more than 7.5; (7) the aqueous solution at a c centration of 30 mg per ml is substantially free of any turbidity undissolved substances.

Disposal of Residues

In the production of penicillin, large quantities of resid waste, consisting of mycelium, extracted broth, and wash wa from equipment, are obtained. These residues have relativ high b.o.d. values and present a serious disposal problem many plants. Heukelekian[26,27] reported that the stripped p icillin beer from one plant had an average b.o.d. of 4,380 pp A significant portion of this b.o.d. was contributed by the resid amyl acetate in the broth. The b.o.d of the wash water from same plant averaged 3,420 ppm. The mycelium also has a h b.o.d.

The methods employed for disposing of the penicillin wa varies from plant to plant. Those which are located in or n large metropolitan areas are able to dispose of the residues throu the sanitary sewage system. However, there are a number of pla in areas where the capacity for sewage disposal is insufficient handle the penicillin wastes. These plants have to dispose of residues by other means, some of which involve considerable pense. Usually the mycelium is handled separately from the tracted broth and wash water. A few plants dry the myceli by drum or spray drying and sell the dried mycelium as a f supplement. This material has found a ready market at a p which at least pays for the cost of drying. Its approximate c position is as follows.

Composition of Dried Penicillium Mycelium

Protein (6.25 × TN)	32.0%
Moisture	8.0
Fat	7.0
Ash	20.0

Crude fiber	7.0
Nitrogen-free extract	26.0
Choline	3,700.0 μg per g
Thiamine	6.0
Riboflavin	37.0
Pantothenic acid	64.0
Nicotinic acid	140.0
Folic acid	7.0
Biotin	5.0
Pyridoxine	13.0
Vitamin B_{12}	0.02

At least one plant combines the mycelium and extracted broth and drys the mixture. Several plants haul the mold to some isolated area and bury it in the ground. The liquid wastes are disposed of by evaporation and burial or by treatment in a sewage-disposal plant installed for this purpose. It is probable that in the next few years, improved methods of handling penicillin wastes will be developed and the recovery of as much material as possible in an economically valuable form will be the general practice in the penicillin industry.

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CHAPTER 8

STREPTOMYCIN

H. B. Woodruff and L. E. McDaniel

Streptomycin is a therapeutic agent which was discovered in the course of a planned survey of microorganisms for antibiotic properties. Early in the survey, several antibiotics having little value as chemotherapeutic agents were obtained at the Department of Microbiology of the New Jersey Agricultural Experiment Station, Rutgers University.[39] Those which have been purified and studied in some detail include actinomycin, clavacin, fumigacin, chaetomin, micromonosporin and streptothricin. Toxicity, in activity *in vivo,* production difficulties, or other factors limited usefulness of these compounds, but the experience gained in their production and isolation, particularly in the case of streptothricin contributed greatly to the speed with which streptomycin was developed from a laboratory agent to a commercially availabl chemotherapeutic compound.

Two microorganisms, one (No. 18-16) from a heavily manured field soil and the other (No. D-1) from the throat of a chicken produced an antibiotic different, in the range of microorganism inhibited, from all previously described antibiotics. High dilution of fermented broths of the cultures inhibited growth of many Gram positive bacteria, Gram-negative bacteria, particularly the coliform and intestinal parasites, and the acid-fast bacteria. The antibioti agent was named *streptomycin.* The antibiotic activity of the partially purified product, isolated from such broths, is now known to be due to a mixture of antibiotics, called the streptomycin complex. A bibliography of the literature on streptomycin through 1952 has appeared.[38b]

The microorganisms which produced streptomycin were similar in cultural characteristics and in morphology to *Actinomyces griseus,* isolated from soil and described 28 years previously. According to modern schemes of classification, the two microorganisms are identified as *Streptomyces griseus.*

The production of streptomycin is a strain-specific characteristic of *S. griseus.* Numerous attempts to isolate streptomycin-producing strains from nature have been made, but descriptions have been published of only a few strains which produce streptomycin in appreciable quantity. Many strains produce a small quantity of inhibitory substance which has not been positively identified as streptomycin. A filter-paper strip-chromatogram method has been used to demonstrate chemical similarity of the inhibitory substance from some of these strains to streptomycin.[16]

Streptomycin is associated with a second antibiotic, possibly streptothricin, in the fermented broth of one strain of *S. griseus.*[38] A substance similar to streptomycin is produced by a culture closely resembling *S. griseolus,* which was given the name *S. bikiniensis* on the basis of minor cultural distinctions.[18]

Many of the cultures which are used at present in industrial organizations for the production of streptomycin originated from one of the original isolates of *S. griseus,* No. 18-16, although new isolates from natural sources are used in some plants.

LABORATORY FERMENTATION METHODS

The original description of streptomycin production indicated that both a shallow-layer stationary method and an aerated submerged procedure were satisfactory for growth of *S. griseus* and for streptomycin production.[34] Various organic complexes are satisfactory as components of media. (See the section on raw materials.) Working details of a method for producing streptomycin by surface culture in pint milk bottles were described in a report from the Wellcome Physiological Research Laboratories.[1] Streptomycin yields in the crude-culture filtrates were about 250 μg per ml after 10 to 14 days of incubation.

Working Details of the Surface-Culture Method

Since the procedures for laboratory and pilot-plant produ
tion of streptomycin are similar, the second is described here.

An important ingredient used in the media is papain dig
of beef which is prepared as follows: 120 lb of beef muscle
placed in 90 l of warm water, brought to 60°C, and 190 g of po
dered papain added. The mixture is incubated at 60°C for
hours and the digest is then boiled 5 minutes, cooled, and filter
After storage at 5°C, the fat on the surface is removed by ski
ming. The spore-production medium contains 6% by volu
papain digest of beef, and by weight, 1.5% molasses. 0.58% glycer
0.5% NaCl, 0.0015% $FeSO_4 \cdot 7H_2O$. 0.009% $CuSO_4 \cdot 5H_2$
0.0013% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.00028% $MnSO_4 \cdot 4H_2O$, 0.006
KH_2PO_4, and 0.005% $MgSO_4 \cdot 7H_2O$, in water. After adjusting
pH 7.0 ± 0.2 with sodium hydroxide, the unfiltered medium
distributed in 300 ml quantites into 2.5 l Roux bottles a
autoclaved.

The stock culture of *S. griseus* (NCTC 7187) is stored
sealing the cotton plugs of agar-slant cultures with liquef
paraffin and holding at 4°C in the dark. Spores from a sin
stock culture of *S. griseus* are transferred to a single flask of spo
production medium. After 7 days incubation at 28.5°C. the gro
pellicle and spores are thoroughly suspended by shaking with
ml of 1% "calsolene" solution and used to inoculate an additio
lot of fifty bottles.

The production medium contains by volume, 5% papain dig
of beef and by weight, 0.4% $NaNO_3$, 0.1% KH_2PO_4. 0.05% K
0.2% sodium citrate, 0.2% sodium acetate, 0.004% $MnSO_4 \cdot 4H$
0.00175% $CuSO_4 \cdot 5H_2O$. 4.0% glucose monohydrate, and 0.2
extract of beef (Wilsons). The medium is filtered with Cel
adjusted to pH 7.0, and refiltered.

Milk-bottling machines are used for filling 200 ml of medi
into pint milk bottles. The bottles are not plugged with cot
but are covered by loose-fitting aluminum caps and autocla
at 15 psi steam pressure for 15 minutes. Extension of autocla
time to 1 hour makes the medium useless for streptom
production.

Inoculation is accomplished by means of spray guns wh istribute about 3 ml of spore inoculum as a fine spray into each int bottle.

Cultures are incubated 10 to 14 days at 28.5° ± 1°C in a anted position in large, movable dollies.

Harvesting is effected by a mechanical harvester in which ottles are inserted and drained of their contents. Empty bottles re transferred to the bottle-washing and medium-filling machine.

Solid Substrate

Moist solid substrates, such as rice bran and wheat bran, are atisfactory for development of heavily sporulated inoculum of *. griseus.* For best results, S. *griseus* requires adaptation to growth n rice bran. Repeated transfer of spores from rice-bran cultures o sterile, moist rice bran eventually results in rapid growth. porulated rice-bran cultures may be used as an inoculum for roduction of streptomycin by the stationary-culture method escribed before.

Streptomycin is produced as a result of growth on a rice-ran medium, but is absorbed on the substrate. The antibiotic nay be released by leaching the cultures with dilute acids dis-olved in water or alcohol. A 0.1 *N* solution of formic acid is a atisfactory leaching agent.

Supplementation of the moist rice-bran medium with 1% odium chloride improves the yield of streptomycin. The addi-ion of water equivalent to three fifths of the weight of the dry ran yields a satisfactory consistency of substrate for growth of . *griseus* in shallow layers in laboratory glassware. In larger olumes, aeration must be maintained at an adequate level, by he use of rotary-drum fermentors or similar methods.

Laboratory yields on a rice-bran substrate averaged 1,400 μg f streptomycin per gram of dry bran in an incubation period of 4 to 21 days.[41] The best yield obtained with the same strain of *griseus* in comparative experiments made with stationary liquid ıltures was 400 to 500 μg of streptomycin per ml of broth, in 9 14 days.

Submerged Cultures

Laboratory studies of the submerged-culture technique are nited mostly to shake-flask cultures.

Details of a successful procedure are as follows. Medium
dispensed in 40 to 80 ml amounts in 250 ml Erlenmeyer flask
The flasks are plugged with cotton and sterilized at 15 psi stea
pressure for 15 to 20 minutes. The medium is inoculated wit
spores of *S. griseus,* removed from an agar culture, or with
portion of a submerged culture, preferably in the logarithm
phase of growth. The flasks are placed on a shaking-machi
platform which rotates in a horizontal plane through a 360° a
of 1.25 in. radius at 220 rpm. The temperature is maintained
28° to 30°C.

With strains of *S. griseus* similar to those used in exampl
described before and liquid media containing enzymic digests
animal proteins or ground plant products, such as soybean mea
yields of 200 to 500 μg of streptomycin per ml of crude cultu
filtrate have been reported, following a 5-day incubation perio
using spore inoculum. The incubation period required is reduc
by 1 to 2 days if 5 to 10% by volume of a vegetative inoculum
used.

RAW MATERIALS

Recently factors affecting streptomycin yields in submerg
culture have been investigated.[14a] Four physiologically differe
strains of *S. griseus* were tested on a variety of media. Streptomyc
yields were quite different from those reported in the literature f
the various media. It was concluded that the medium must
specifically designed for the strain if the streptomycin-produci
capacity of the organism is to be fully realized. Streptomyc
production from media containing peptone, meat extract, gluco
and sodium chloride was studied systematically. The antibio
yield was markedly affected by the concentration of the mediu
ingredients and by use of tap water. Over 1,200 units of strep
mycin per ml were obtained when the selected highest-yieldi
strain was grown on a medium containing 1% peptone, 0.25
meat extract, 1% glucose, and 0.5% sodium chloride in tap wat

A streptomycin-producing "precursor," has been postulated
Beef extract and corn-steep liquor were reported to contain t
factor, which has been called the "activity factor," in contrad
tinction to the term "growth factor." These results have not be
reconciled with the equivalent production of streptomycin in s

thetic medium (see later). In some media, inorganic salts substitute for beef extract. (Swift's meat extract has been found to contain 35% ash.)

Minor modifications in the protein hydrolyzate included in the medium have considerable influence on streptomycin yields in stationary culture. In addition to Difco peptone and papain digest of beef, described before, Bacto tryptose, Armour beef granules, and yeast extract are satisfactory nitrogen sources, in the presence of beef extract. Other nitrogen sources, including Sheffield N-Z amine, Armour peptonum siccum, and acid-hydrolyzed casein, were found to be unsatisfactory for surface-culture growth of *S. griseus,* because submerged, wet pellicles developed. However, these nitrogen sources were all satisfactory for submerged production of streptomycin.[41]

Supplementing the nitrogenous components, carbohydrates may be supplied in the form of glucose or starch. Glycerol also is satisfactory. Sucrose is not utilized by *S. griseus.* Various glycerides, including lard oil and soybean oil, support normal streptomycin production, when used as a complete replacement for carbohydrates.[27b] Those salts which are essential nutrients must be supplied. Best yields of streptomycin have been obtained in the presence of limited phosphate-ion concentration. Soluble phosphate disappears from the medium during mycelial growth and returns following lysis. If soluble phosphate is present throughout the fermentation, mycelial growth is heavy, but streptomycin production is poor.[12]

Inexpensive media have been devised. Corn-steep liquor is suitable for streptomycin production, but complicates the extraction procedure. Soybean meal, dried fermentation solubles, dried distillers' solubles, groundnut-cake hydrolyzate, or combinations of these satisfactorily serve as the sole source of nitrogen. Acid-hydrolyzed stillage from wheat mash and asparagus-butt juice are satisfactory in a medium supplemented with corn-steep liquor ash. Certain fermentation ingredients which have been found valuable in commercial production have been claimed in patents.[7,10,23,24,25,37] Two examples of media which have proved satisfactory for streptomycin production in submerged culture are listed as follows.

Medium A: 1% glucose, 0.5% peptone, 0.5% meat extract, 0.5% sodium chloride.

Medium B: 1% soybean meal, 1% glucose, 0.5% sodium chloride.[30]

S. griseus grows well in certain media containing inorganic sources of nitrogen, provided the media are balanced to maintain the pH in a range suited for growth. Usually, streptomycin production is poor in such media. *S. griseus* does not utilize nitrate nitrogen in the absence of a supplemental nitrogen supply. Ammonium lactate or diammonium phosphate serve as satisfactory nitroge sources, since the complete compounds are assimilated. Yields of 150 μg per ml have been obtained from growth of *S. griseus* by the submerged method on a medium containing 10 g glucose 2 g NaCl, 2 g K_2HPO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.4 g $CaCl_2$, 20 mg $FeSO_4 \cdot 7H_2O$, 4 g $(NH_4)_2HPO_4$, and 1 l distilled water.

A number of amino acids may be utilized for growth of S *griseus.* Only six, DL-α-alanine, β-alanine, L-histidine, glycine L-proline, and L-arginine supported yields in excess of 100 μg pe ml. Striking results have been obtained with L-proline as a sol nitrogen source. Growth of the culture was heavy and yields o 800 μg per ml were obtained with the same strain which gave th yields in organic media described in the section on laborator procedures.[11] There is a long lag before initiation of growth o *S. griseus* on a proline medium, but the lag may be reduced som what without impairing streptomycin production if a trace o ammonium nitrogen is present.

In a synthetic medium containing glucose, sodium lactat ammonium, and phosphate, elimination of potassium, magnesiu or zinc limits growth. Iron, magnesium, and copper have no e fect on growth when each is separately eliminated from the mediu All these elements, except copper, are required for maximu streptomycin production. The optimum molal levels of salts each element for streptomycin production in the synthetic mediu were found to be: 0.01 disodium phosphate, 0.06 potassium chlorid 0.01 magnesium sulfate, 0.00005 zinc sulfate, 0.00005 ferrous sulfat and 0.0005 manganous chloride.

Other trace elements, when added to the before-mention synthetic medium, either singly or in combination, did not fect production or growth. However, certain elements, when add to a synthetic medium which contained no Zn, Fe, Mn, and C influenced growth and streptomycin production. The elemen studied were Co, Mo, As, Hg, Pb, W, Bi, U, Be, Ti, V, Sr, Th, Ba, B, Cr, I and Sb.[36]

A mixture of known vitamins did not affect streptomycin production. It seems probable that the "activity factor," necessary to promote streptomycin formation, is inorganic in nature. A proper concentration of corn-steep ash or potassium-bearing salts, such as potassium phosphate or chloride, has been reported to replace beef extract in media containing protein hydrolyzates.[5] In a medium containing soybean meal, either sodium chloride or sodium sulfate are essential ash constituents and may be supplied by beef extract.

FERMENTATION MECHANISM

Complex Media

The knowledge of metabolic activities of the actinomycetes in submerged or surface culture is quite limited. The chemical changes which take place in submerged fermentation of glucose-meat extract-peptone medium have been measured[12] and are given in Table 33. The fermentation falls into two fairly distinct phases. During the first stage, the mycelium is formed. There is a decrease in soluble nitrogen and carbon in the medium and utilization of glucose and inorganic phosphate is practically complete. The lactic acid content of the medium at first increases and then is fermented. The formation of streptomycin begins.

TABLE 33. CHANGES OCCURRING DURING FERMENTATION OF A GLUCOSE-MEAT EXTRACT-PEPTONE MEDIUM[a]

	Duration of fermentation, days								
	0	1	2	3	4	5	6	7	8
pH	7.35	7.30	7.55	7.50	7.75	8.25	8.55	8.65	8.90
Mycelium, mg per ml	—	0.4	5.1	5.8	5.7	4.8	4.6	4.2	3.8
Streptomycin, μg per ml	—	0	37	194	198	231	270	186	267
Glucose, mg per ml	9.0	8.8	8.0	2.4	1.2	0.6	—	—	—
Soluble carbon, mg per ml	10.2	8.6	7.0	5.1	5.0	4.4	4.6	4.5	4.6
Lactic acid, μg per ml	292	328	114	13	10	16	12	6	15
Oxygen demand, Q_{O_2} per ml	—	19	81	82	53	25	—5	—	—
Soluble nitrogen, mg per ml	1.48	1.30	1.10	0.67	0.70	0.73	0.90	0.88	1.14
Mycelial nitrogen, mg per ml	—	0.04	0.44	0.62	0.57	0.49	0.40	0.38	0.29
Inorganic phosphorus, mg per ml	118	108	34	1	5	2	19	24	34
Ammonia nitrogen, μg per ml	66	70	75	63	103	115	170	232	265

[a] Medium: 10 g glucose, 5 g meat extract, 5 g peptone, 5 g sodium chloride per liter.

During the second phase, streptomycin accumulates. There is a rise in pH, accompanied by lysis of the culture. Soluble nutrients increase as a result of liberation during autolysis of cells.

Continued incubation of the culture after lysis has occurred leads to a decrease in streptomycin titer in the broth. The cause of the decrease has not been determined. Although streptomycin is exceptionally resistant to enzymic decomposition by soil microorganisms and probably is not destroyed by enzymic action of lytic products, it has been inactivated by incubation with certain microorganisms isolated from streptomycin-enriched soils.[29]

C-14-labeled glucose and starch have been used for the preparation of C-14-labeled streptomycin by laboratory-scale fermentation.[1] The labeled product is useful in studying the mechanism of actio of streptomycin and in the development of isotopic dilution assa for the antibiotic. Studies with a fermentation medium containin glucose and C-14-labeled glycine and acetate showed that th streptomycin carbons were derived almost entirely from glucos The small amount of labeled carbon found in the streptomyci was located in the guanidine carbons.[25a]

Oxygen Tension

The oxygen demand of submerged cultures of *S. griseus* high, not only during the logarithmic phase of mycelial growt but also during the rather extensive period in which myceliu weight remains constant, previous to lysis. In glucose-meat extrac peptone media of varying composition, the Q_{O_2} per ml (micr liters of oxygen taken up per milliliter of culture per hour) reache a plateau at 80 to 120.[12] This demand is reflected in a fall i oxidation-reduction potential of the medium to a constant valu as a result of the growth of *S. griseus*.

Aeration of uninoculated medium at rates of 0.5 to 6 volum per minute stabilizes the oxidation-reduction potential of th medium at 310 to 375 mv, regardless of the rate. Oxidation-redu tion measurements of laboratory cultures of *S. griseus* vari directly in relation to the before-mentioned aeration rates. Th different rates had a marked effect on the fermentation proce The pH was higher at high air rates, possibly indicating that th fermentation had proceeded further. Also, about 50% more stre tomycin was produced at the higher air rates.[20]

A quantitative study of oxygen transfer and agitation in 5-l laboratory-scale fermentors provided data and techniques which were used with success in translation among laboratory-scale, pilot-plant, and factory fermentors.[3] The data fit a diffusion theory, which was expressed mathematically. Values for the rate of oxygen uptake by the organisms calculated from the equations agreed well with the experimentally determined values.

The rate of oxygen absorption by *S. griseus* cells is independent of the oxygen concentration at the cell-liquid interface if the concentration exceeds about 10^{-8} gram mole of oxygen per ml.

Streptomycin production was found to be related to power input and air-flow rate. It increased with increase in power to a broad maximum, ultimately decreasing at high agitator speed. Productivity with sintered spargers was greater than with constricted pipe spargers. Air-flow rate was also important, but the critical level was dependent on agitator speed. An optimum rate of oxygen uptake at the cell surface is obtained with constant rates of cell growth and antibiotic production above threshold values of k_a (oxygen-transfer coefficient) and of power absorption. The combinations of air-flow rates and mechanical-power input to achieve or exceed these threshold levels are the basis for economic fermentor design.

CULTURE SELECTION AND MAINTENANCE

S. griseus is a very unstable microorganism. Its variants differ in their ability to yield streptomycin. Morphological differences between single-colony isolates are also apparent. Substrains differ in degree of sporulation, soluble-pigment production, and types of growth. Some variants differ so much that they do not fit in the generic classification *Streptomyces*, but more closely resemble *Nocardia*. Old, giant colonies show distinct sectoring. While variation presents a problem in maintenance of stock cultures, use has been made of it to isolate strains of greater productivity. Strains of improved productivity have been selected from natural variants. nduced mutation with ultraviolet light, followed by single-colony election, has resulted in isolation of strains which yield over 900 g per ml of streptomycin on ordinary plant-production media.[37] Recently Pittenger and McCoy[27c] studied variants of *S. griseus* nduced by ultraviolet irradiation. Five of 98 strains selected for high activity from more than 4,000 colonies screened gave highe submerged-culture yields of streptomycin than the parent cultur One of these five strains excelled the parent in all four med used. Variation in streptomycin production on the different med by an individual strain points up the fact that the choice of single medium for screening may restrict the detection of hig yielding strains.

Cultures may be stabilized by lyophilization procedures, described in Chapter 12. Spores of active cultures, suspended sterile skimmed milk, form an ideal pellet on lyophilization. Te for activity, growth, spore production, and physical appearan showed no difference between the parent and lyophilized culture An alternate method, described in some detail in Chapter 12, the preparation of soil cultures. Spores of *S. griseus* are mix with sterile soil and allowed to dry out at room temperature. T stock soil cultures are satisfactory for development of inoculu for experimental purposes.

DEVELOPMENT OF CULTURE

S. griseus is developed for initiation of production batches aseptic transfer of lyophile pellets or grains of soil cultures to nutritive medium. A satisfactory sporulation medium is describ in the section on procedures for the surface-culture method. medium which is adequate for initiating submerged growth c tains 1% yeast extract, 1% glucose, and 0.5% $Na_2HPO_4 \cdot KH_2P$ buffer of pH 7.0.

PLANT FERMENTATION

Description of the submerged, aerated production process e ployed at Merck & Co., including a flow sheet, has been publishe Campbell[5a] has also presented a flow sheet for the manufacture streptomycin. Details of the Merck production process are su marized here, and the flow sheet is given in Figures 19a to 19d.

An agar slant, inoculated with a large loop of lyophilized culture of *S. griseus*, is incubated until heavy sporulation occ Spores are suspended in water and transferred to initiate growth three 300-ml shake-flask cultures by incubation as described in section on submerged-culture laboratory procedures. The

FIGURES 19a-19d. *Flow Diagram of Streptomycin Production and Recovery Process* [Reprinted by permission from *Chem. Eng.*, **53,** No. 10, 142 (1946)]

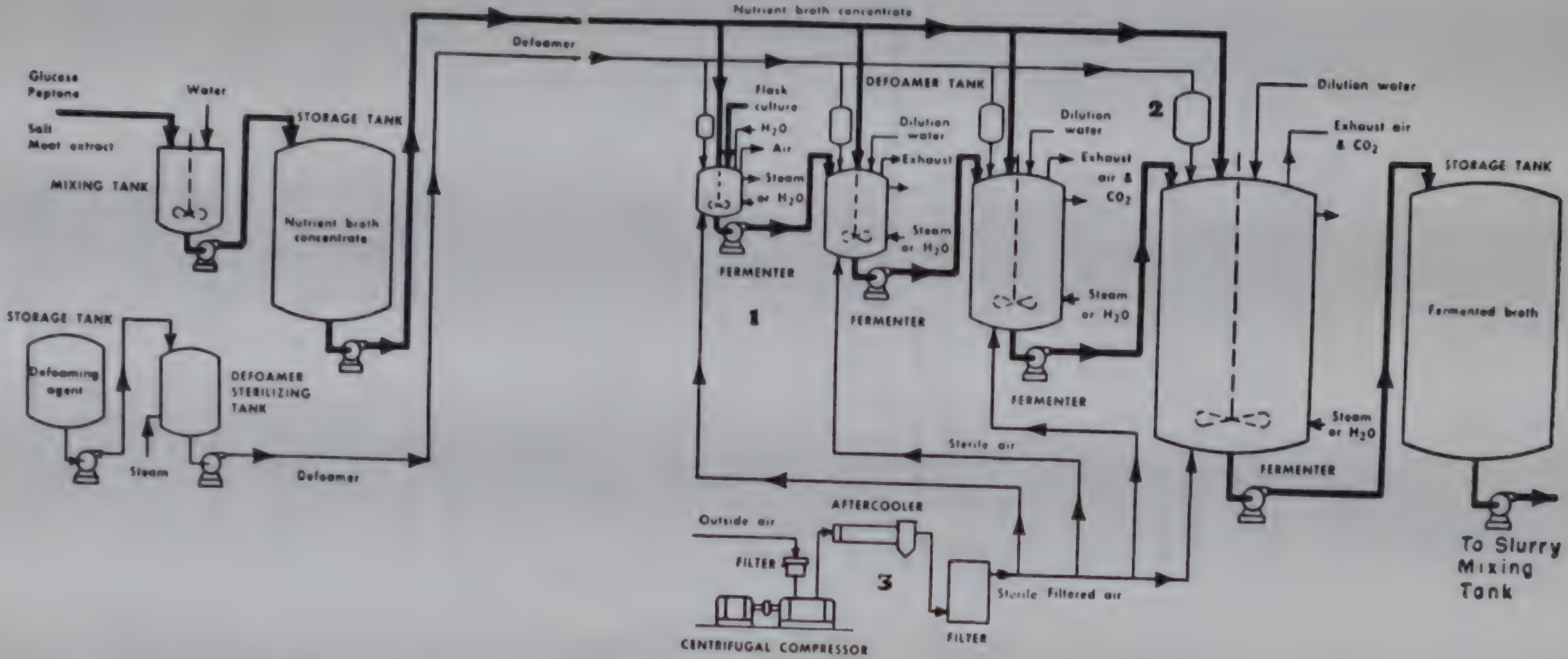

Glucose
Peptone
Salt
Meat extract
Water
MIXING TANK
STORAGE TANK
Nutrient broth concentrate
STORAGE TANK
Defoaming agent
Steam
DEFOAMER STERILIZING TANK
Defoamer
Nutrient broth concentrate
Defoamer
Flask culture
H_2O
Air
Steam or H_2O
FERMENTER
1
DEFOAMER TANK
Dilution water
Exhaust
Steam or H_2O
FERMENTER
Dilution water
Exhaust air & CO_2
Steam or H_2O
FERMENTER
2
Dilution water
Exhaust air & CO_2
Steam or H_2O
FERMENTER
STORAGE TANK
Fermented broth
To Slurry Mixing Tank
Sterile air
Outside air
FILTER
AFTERCOOLER
3
CENTRIFUGAL COMPRESSOR
FILTER
Sterile Filtered air

FIGURE 19a. *Medium Preparation and Fermentation*

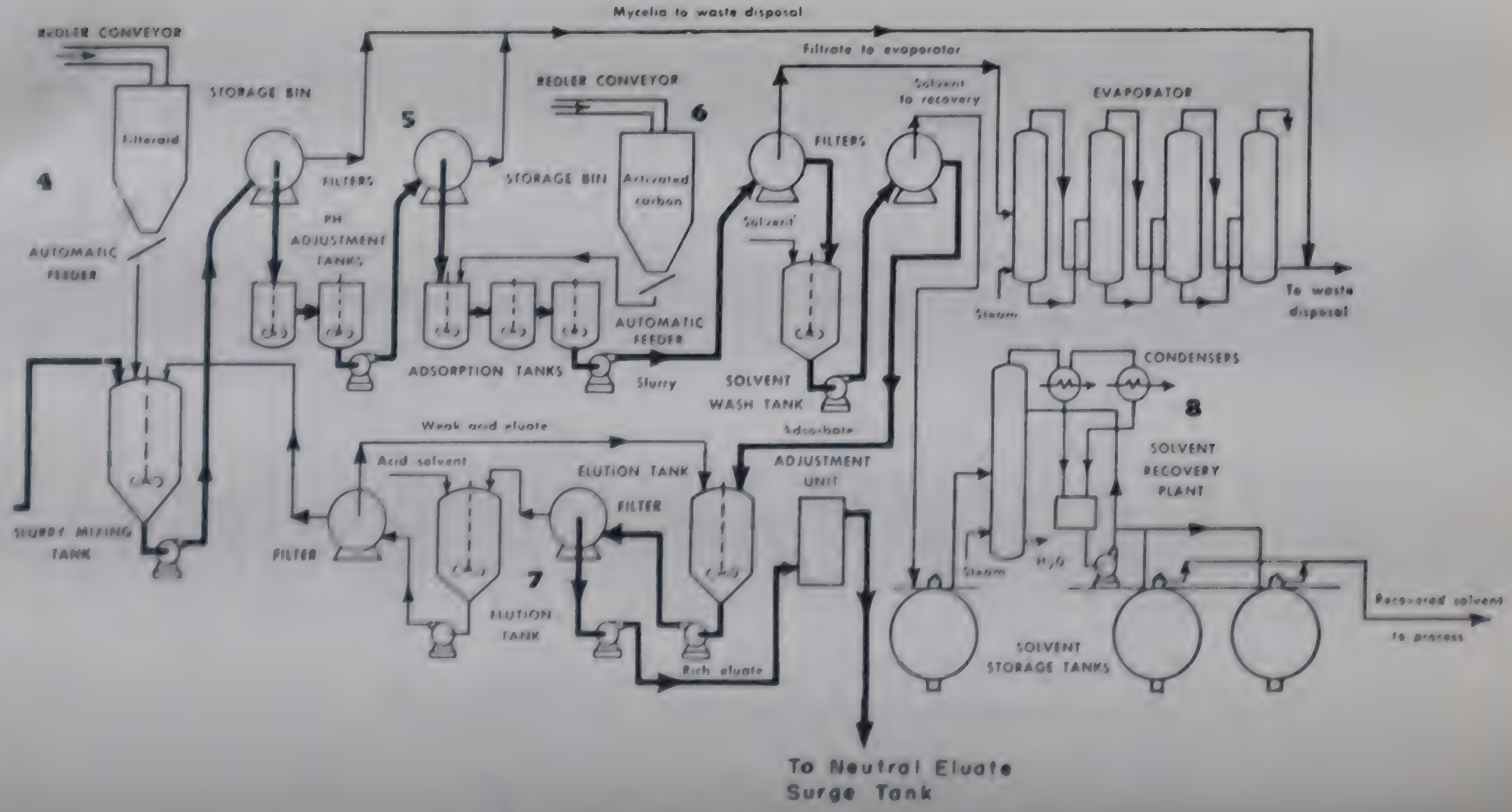

Mycelia to waste disposal
REDLER CONVEYOR
4
Filteraid
STORAGE BIN
AUTOMATIC FEEDER
SLURRY MIXING TANK
FILTERS
PH ADJUSTMENT TANKS
5
REDLER CONVEYOR
6
STORAGE BIN
Activated carbon
AUTOMATIC FEEDER
ADSORPTION TANKS
Slurry
Filtrate to evaporator
Solvent to recovery
FILTERS
Solvent
SOLVENT WASH TANK
Adsorbate
EVAPORATOR
Steam
To waste disposal
Weak acid eluate
Acid solvent
FILTER
7
ELUTION TANK
ELUTION TANK
FILTER
Rich eluate
ADJUSTMENT UNIT
To Neutral Eluate Surge Tank
CONDENSERS
8
SOLVENT RECOVERY PLANT
Steam
H_2O
SOLVENT STORAGE TANKS
Recovered solvent to process

FIGURE 19b. Adsorption and Elution

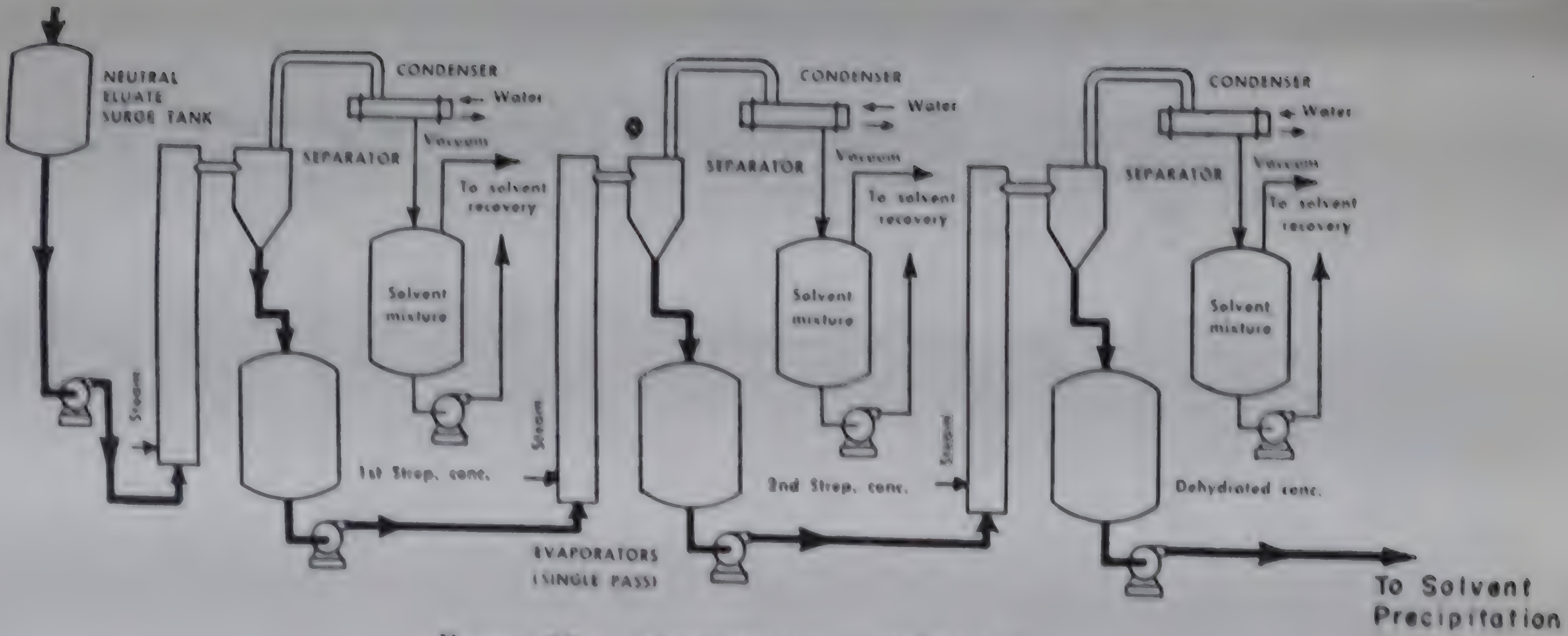

NEUTRAL ELUATE SURGE TANK
CONDENSER
Water
SEPARATOR
Vacuum
To solvent recovery
Solvent mixture
Steam
1st Strep. conc.
EVAPORATORS (SINGLE PASS)
CONDENSER
Water
SEPARATOR
Vacuum
To solvent recovery
Solvent mixture
Steam
2nd Strep. conc.
CONDENSER
Water
SEPARATOR
Vacuum
To solvent recovery
Solvent mixture
Steam
Dehydrated conc.
To Solvent Precipitation

FIGURE 19c. *Concentration and Dehydration*

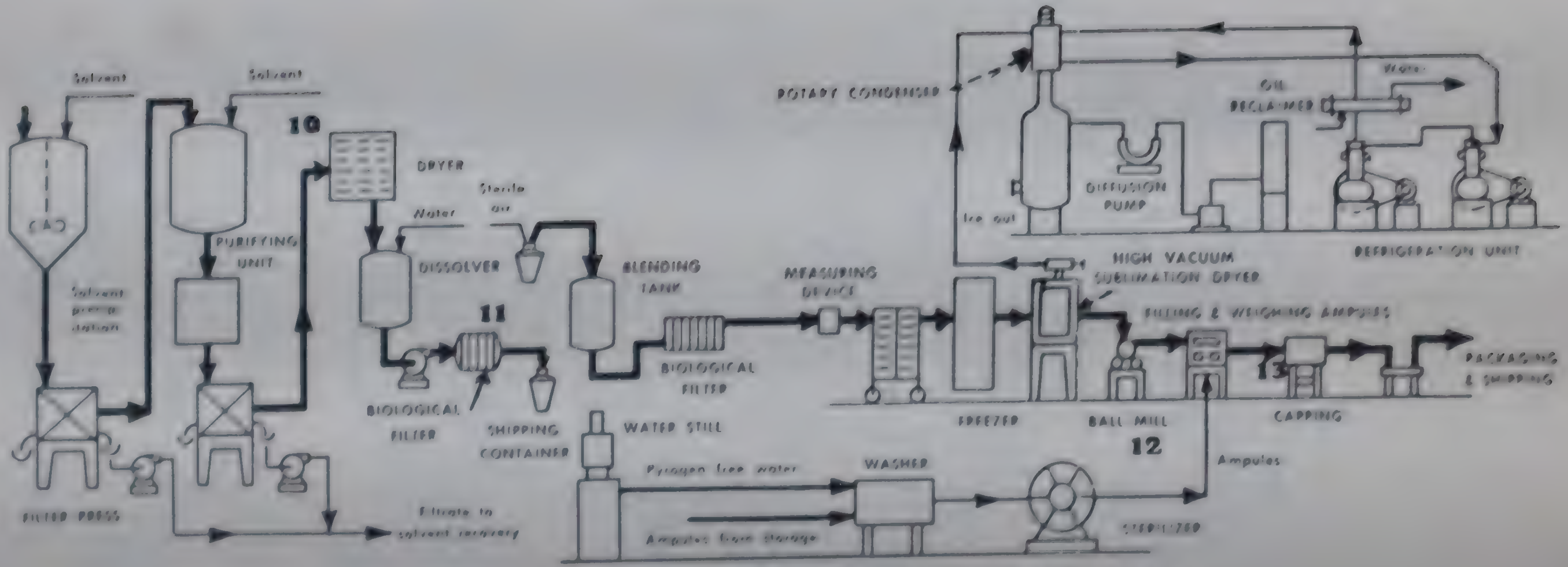

Solvent
Solvent
10
DRYER
(A)
Sterile air
Water
PURIFYING UNIT
DISSOLVER
Solvent precipitation
11
BIOLOGICAL FILTER
SHIPPING CONTAINER
FILTER PRESS
Filtrate to solvent recovery
BLENDING TANK
BIOLOGICAL FILTER
WATER STILL
Pyrogen free water
Ampules from storage
WASHER
MEASURING DEVICE
ROTARY CONDENSER
Ice out
DIFFUSION PUMP
OIL RECLAIMER
Water
REFRIGERATION UNIT
HIGH VACUUM SUBLIMATION DRYER
FILLING & WEIGHING AMPULES
FREEZER
BALL MILL
12
13
CAPPING
PACKAGING & SHIPPING
Ampules
STERILIZER

FIGURE 19d. *Precipitation, Purification, and Finishing*

flask culture becomes the starting point for mass produc-
he plant.

materials of the nutrient medium are mixed with water
centration about ten times higher than finally required.
h is pumped either through a continuous sterilizer or di-
fermentors where, properly diluted, it is sterilized and

r sizes of fermentors, constructed of carbon steel, are em-
for fermentation. Each vessel in the series was designed
ide culture for efficient inoculation of the next larger
or in the series. The laboratory culture serves as inoculant
smallest tank. During fermentation, the temperature is
controlled at 25° to 30°C by cooling with water. Total
ation time for all four vessels in series amounts to several
though each stage follows its own assigned schedule. The
of inoculant tanks is such that maximum fermentor
is always in operation.

ation is accomplished by air from sterilizing filters, sup-
the tanks at approximately 0.03 cu ft per minute per gal
entor capacity. Each vessel is equipped with an agitator
ote aeration.

a result of aeration, considerable foaming occurs. This is
ed by the addition of sterile antifoam agents, which must
ted for lack of toxicity.

e final fermented culture volume is about 15,000 gal. The
ycin content of broths produced commercially has not
ublished. Laboratory and pilot-plant fermentor yields
from 150 to 1,200 μg per ml in production-type media have
ported.

mentation operations must, at all times, be conducted
septic conditions to prevent contamination, which might
or reduce streptomycin production or add to extraction
es. All joints and connections to the fermentor are pro-
y steam seals. Valves are steam sealed and all pipes lead-
he fermentor are kept under steam pressure except when
Streptomycin is not subject to destruction by the usual
bacterial contamination, so that streptomycin produced
he appearance of contaminants will not be lost.

ddition to precautions against bacterial contamination,
essary to guard against phage infestations. Lysis by actino-

phage is particularly serious when inoculum vessels are inva
since the inoculum becomes useless for initiating fermentatio
the production fermentors. Actinophage infection, initiate
the production tank, leads to early lysis of the *S. griseus* an
duced yields. Carvajal[6a] recently described an extensive stud
phage problems in the streptomycin fermentation. He rep
that in one manufacturing plant during a 3-week period, 7
111 fermentors were infected with a multivalent phage. Effe
measures for control and eradication depended on strict san
precautions and mainly on development of cultures immun
action of the phage. Actinophage-resistant strains of *S. g*
have been developed which retain the ability to produce str
mycin and are used in factory processes.[6a,33,42]

Isolation details, as well as the production process, are
grammed in the flow sheet of Figures 19a to 19d. A series o
operations is used: (1) removal of mycelium by filtration,
adsorption of streptomycin on activated carbon, (3) elutio
streptomycin from the carbon adsorbate, (4) concentration o
eluate by evaporation and dehydration, (5) double solvent
cipitation, followed by filtration and drying. Further purific
is accomplished by undisclosed procedures to yield a produ
over 90% purity.

Activated carbon is a relatively nonspecific adsorbent a
great deal of other substances becomes adsorbed and eluted
the streptomycin. The procedure may be made more speci
treating streptomycin-containing material with carbon at pH
Impurities are adsorbed, but most of the streptomycin is not
recovered in a purified state. Adsorbed streptomycin is elute
reworked.[22]

Streptomycin has been purified by alumina chromatog
and crystallized with a number of reagents. Crystalline st
mycin reineckate and streptomycin helianthate have been des
and procedures given for conversion to pure but noncryst
streptomycin hydrochloride. A crystalline streptomycin tri
chloride-calcium chloride double salt has been made. Because
purity, this salt has advantages for clinical application an
marketed in lyophilized form as the final product from the
described previously.

An example of recovery and product purity obtained b
ous purification steps is shown in Table 34. These data

TABLE 34. REPRESENTATIVE LABORATORY PURIFICATION PROCEDURES

Experiment	Steps	Weight Recovery, g		Recovery of activity, %	Activity, μg per mg
		Taken	Recovered		
No. 1[a]	*a.* Broth	10.5 (liters)	—	—	82
	b. Acetone ppt from eluate of pH 8 adsorption	—	—	—	30-80
	c. Acetone ppt from eluate of pH 4 adsorption of *b*	—	1.010	11	60-85 (reworked to step *d*)
	d. Unadsorbed portion of step *c* adjusted to pH 6. Acetone ppt of eluate of charcoal adsorption at pH 6	—	0.475	22	350-450
No. 2[b]	*a.* Streptomycin hydrochloride absorbed on 160 g alumina	6.0	—	—	225
	b. First 250 ml methanol elute	—	0.698	23	300
	c. Next 150 ml elute	—	1.031	34	490
	d. Next 250 ml elute	—	0.729	14	290
	e. Next 350 ml elute	—	0.413	4	150
No. 3	*a.* 500 μg/ml streptomycin in methanol solution crystallized with 20.8 g methyl orange	26.3	32.000	87	—
No. 4[c]	*a.* 7 g $CaCl_2$ in methanol added to methanol solution of streptomycin helianthate. Filtrate concentrated and crystallized	30.0	11.500	77	750

[a] LePage and Campbell.[22] [b] Kuehl, Peck, Hoffhine, Graber, and Folkers.[21] [c] Peck, Brink, Kuehl, Flynn, Walti, and Folkers.[20]

been selected from various published laboratory experiments a probably are representative of yields which may be expected fr similar processes on a production scale.

A more recent development is the application of ion-excha methods to the purification of certain basic antibiotics. This velopment is of great practical value in the commercial prepa tion of streptomycin. Ion-exchange resins had been suggested several occasions as adsorbents for streptomycin, but had not be altogether satisfactory because the resins adsorbed not only antibiotic but various other complex organic compounds inv ably found in fermentation broths. Thus, as with activated carb the adsorption step served primarily as a concentration step a which numerous complicated purification steps were required final purification.

Ion-exchange resins having carboxylic polar groups h found widespread application for the adsorption of streptomy The product obtained directly from the broth by use of carbox acid resins is of such purity as to yield directly a crystalline cium chloride complex salt from methanol solution. Nonb substances are not adsorbed because of the ion-exchange natur the adsorption. The resins are also highly selective for stre mycin among the various organic ions contained in the ferme tion broth. The adsorbed streptomycin is eluted from the r by practically stoichiometric amounts of acid. It is possible recover the streptomycin in almost quantitative amounts from fermentation broth. The resin may be converted to the basic c and reutilized indefinitely.

Characteristics of the adsorption with various adsorbents reported in Table 35.

Various technical modifications have been proposed for ficient use of ion-exchange resins. They are usually packe columns arranged in series to provide a continuous-flow proce

An example of a laboratory scheme, which is adaptabl plant operation, is quoted from the patent of Howe and Put as follows:

> Two columns containing 50 g of a granular copolym methacrylic acid and divinyl benzene containing 5% di benzene were converted to the sodium cycle by passing thr each column 1,000 cc of 10% aqueous sodium hydroxide. columns were washed with 2,000 cc of water each.

TABLE 35. COMPARISON OF VARIOUS ION-EXCHANGE RESINS AND ADSORBENTS IN THE RECOVERY OF STREPTOMYCIN

Type of adsorbent	Capacity of adsorbent mg per g	Eluting agent used	Recovery on elution %	Potency of product obtained units per mg[a]
Sulfonated phenol-formaldehyde resin	10	Aqueous NaCl Aqueous H_2SO_4	30-50 40	} ca 100
Silicate-exchange resin	40	Aqueous NaCl	100	ca 50
Sulfonated coal resin	12	Aqueous NaCl Aqueous H_2SO_4	40-60 40	} ca 100
Carbon	20	Acidic menthanol-water	60-75	200-350
Fuller's earth	50	Aqueous pyridine hydrochloride	30-40	75
Copolymer of methacrylic acid and divinyl benzene, containing 5% divinyl benzene	600-1,500	Aqueous HCl	100	600
Copolymer of methacrylic acid and divinyl benzene, containing 10% divinyl benzene	300-800	Aqueous HCl	100	600
Carboxylic resin believed to be of the phenol-formaldehyde type known as Permutit XHIC	500-900	Aqueous HCl	100	650

[a] Potency for pure streptomycin calcium chloride complex = 779 units per mg.
Source: Howe and Putter.[17]

columns were connected with rubber tubing to operat series flow.

Sixty-four and eight-tenths liters of streptomycin b was acidified to pH 2 with phosphoric acid, filtered, neutra to pH 7 with aqueous sodium hydroxide, and refiltered. treated broth assayed 360 units per cc.

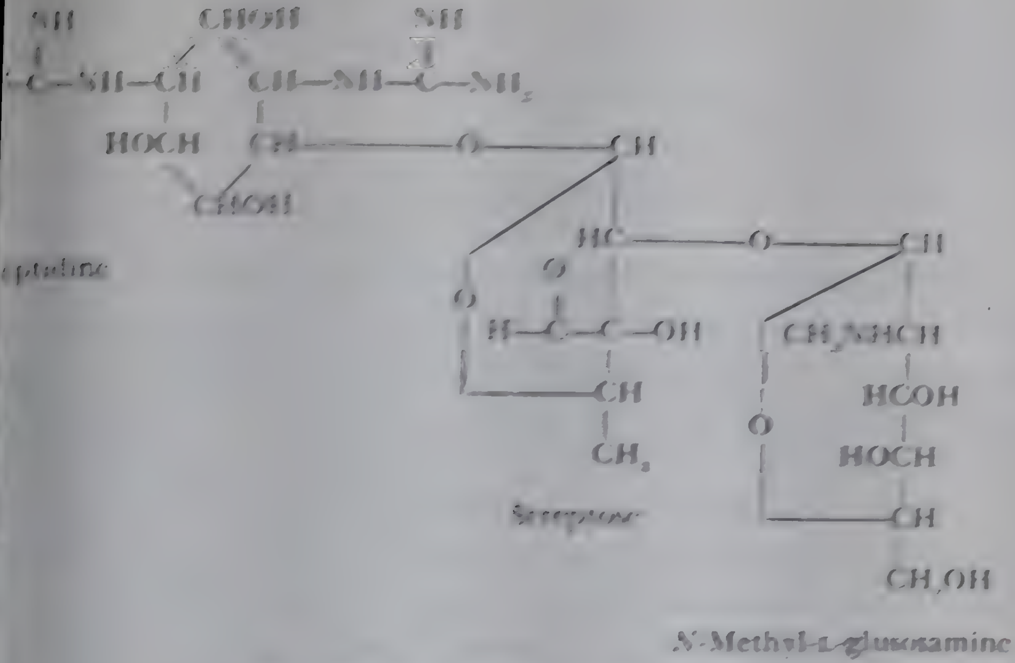
The treated broth was then allowed to flow under a 1 lb pressure through the two resin columns in series rate of 150 cc/min. The spent broth at the end of the assayed 4.8 u./cc. or 1.2%. The columns were washed 1,000 cc of water and then eluted by passing through column 1,000 cc of 1.0 N aqueous hydrochloric acid. The eluate from the first column amounted to 670 cc and as 25,000 u./cc. The rich eluate from the second co amounted to 235 cc and assayed 28,000 u./cc. The rec of streptomycin calculated 100.5%.

The eluate from the first column was concentrated in v at 30°C almost to dryness, treated with 100 cc methanol filtered. The solid removed by filtration weighed 2.75 g. methanol solution was precipitated in 500 cc of aceton solid filtered, washed with 50 cc acetone, and dried in v Weight: 26.8 g, assay: 650 u./mg, ash (sulfated): 15%. recovery at this point calculated 101.5%.

The acetone precipitated solid was dissolved in 81 methanol and to the solution was added 23.6 g of ca chloride, anhydrous. The solution was stirred for 40 and then filtered. The precipitate was washed with a solution of calcium chloride in methanol, then with et and dried. The product weighed 17.5 g and assayed 780 (theoretical for pure streptomycin calcium chloride plex—779 u./mg). The over-all recovery from broth (th the first column) was 80%.

CHEMICAL STRUCTURE

The chemical structure of streptomycin is known, alt the steric arrangement of the molecule has not been comp established. Synthesis by chemical means has not been plished. Streptomycin hydrochloride has the empirical fo $C_{21}H_{39}N_7O_{12} \cdot 3HCl$. It is a glycoside of the inositol deri streptidine, with streptobiosamine, a disaccharide compo the sugars streptose and *N*-methyl-L-glucosamine.



Structure of streptomycin

RELATED THERAPEUTIC COMPOUNDS

reductive hydrogenation, streptomycin is converted to di- treptomycin.[27] This compound has properties similar to mycin, but has a somewhat modified inhibition spectrum microorganisms and has a modified quantitative toxicity se. Most chemicals which form addition compounds on the yl group destroy the antibiotic properties of streptomycin. *griseus* produces a derivative of streptomycin, mannosido- mycin, in some types of media. This derivative is a glycoside tidine with a trisaccharide, composed of streptose, *N*-methyl- samine, and mannose. Against many bacteria it has re- antibacterial effect.[31]

n enzyme is produced late in the fermentation which converts sidostreptomycin to streptomycin. Cultures which are rich me can be used to hydrolyze the mannose from the manno- ptomycin contained in poor fermentations, or that isolated -product in the streptomycin isolation procedure. Strepto- is formed as the end product of the enzymic reaction and an ed fermentation yield is obtained. The reaction may be out by whole cultures of *S. griseus* or by cell-free extracts enzyme.[31a]

other related compound, hydroxystreptomycin, is produced *ptomyces griseo-carneus.* The chemical structure of hydroxy-

streptomycin is similar to that of streptomycin but there is
additional hydroxyl group on the terminal carbon of the stre
portion of the molecule.[4]

PRODUCTION VOLUMES

From the time that streptomycin first became available i
preciable quantity, in August 1946, the production rapidl
creased. Through December 1948, the production curve foll
a constant slope increase, when plotted as a logarithmic
tion against time. This is characteristic of an expanding i
try which has not been subjected to limitations of sale,
materials, or construction restrictions. The basic level of
duction for the United States was at about 8,000 kg per n
for the next 2 years, but domestic consumption has continu
increase. Production of streptomycin for 1951 and 195
159,500 and 175,100 kg. respectively.[7a]

Production data compiled by the U.S. Department of
merce[9] are shown in Figure 20.

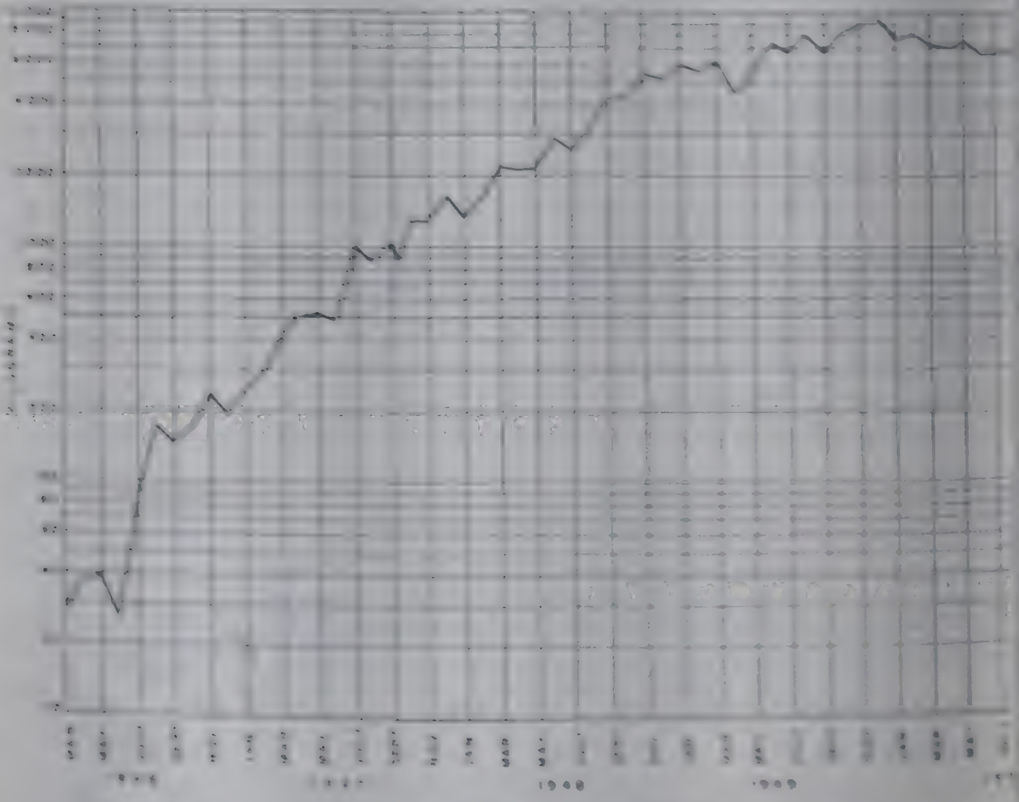


FIGURE 20. *Streptomycin Production by Months*

RESIDUES AND WASTES

e chief residues from the streptomycin fermentation fall
o groups. The first to be obtained is the mycelium of S.
and the insoluble residues from the medium, which are
d during the filtration stage, before the adsorption of
mycin. These residues have potential value as animal feeds.
us mycelium is rich in proteins, being equivalent to the
a in this respect. Although S. griseus does not require an
ous source of B vitamins for growth, it does synthesize ap-
ble quantities of these substances, in this manner adding
value of the mycelial residues as an animal feed.

e degree of contamination of the mycelium with insoluble
s is dependent on the fermentation medium. *S. griseus* has
proteolytic activity and, even in media prepared from
l soybean meal, very little insoluble matter remains at the
tion of fermentation. Because of the bacterial nature of
l growth, much filter aid must be used to obtain a good
on rate. The filter aid contaminates the mycelial wastes
ust be removed to make possible utilization of the residue.

llowing adsorption of streptomycin, the spent broth con-
pproximately 2% soluble organic matter, which can be re-
d by drying, particularly if disposal of high b.o.d. wastes
t a problem. It is useless as a major component of animal
because of its high salt content. With the media which
een proposed for large-scale production of streptomycin, the
ntent of the total soluble solids is greater than 25%. It can,
er, serve as a source material for isolation of the B vitamins,
lly vitamin B_{12}.

mmercially, the most important vitamin by-product of *S.*
fermentations is vitamin B_{12}.[16,22] Some modifications of
ture medium, which have no bearing on streptomycin pro-
, are often necessary to obtain optimum by-product yields.
n B_{12} contains cobalt and, since the natural proteinaceous
used for streptomycin production are often deficient in
the medium is supplemented with a soluble cobalt salt
ncentration just under that toxic for streptomycin produc-
Vitamin B_{12} may be isolated as a pure compound for its
utic properties, or the dried fermentation residues or par-
urified concentrates may be marketed as an animal-feed

supplement for their APF (animal-protein factor) activity. So feed supplements are prepared from combinations of a B_{12}-c taining fermentation residue and an antibiotic to obtain increa growth response and a degree of protection against infection. (also Chapter 6.)

SPECIFICATIONS FOR COMMERCIAL STREPTOMYCI

The specifications for commercial streptomycin have b outlined and are controlled by the Food and Drug Adminis tion[13] of the United States Government. Tests and assays performed on all lots for potency, pyrogens, toxicity, moist pH, histamine-like substances and clarity of solution. Stand methods have been developed for all tests. Potency is measu by biological test for the antibiotic activity of streptomycin aga a standard strain of *Bacillus subtilis* by a cup-diffusion met or *Klebsiella pneumoniae* by a turbidimetric method of assay. potency of streptomycin preparations is designated as the num of "micrograms" (potency unit) contained in a milligram or liliter of sample. A microgram is defined as the streptomycin tivity (potency) contained in 1.38 μg of a streptomycin-calc chloride complex master standard established by the Food Drug Administration. Chemical procedures based on m formation as a degradation product of streptomycin or po graphic analysis are widely used as supplementary analytical cedures. Carboxylic acid ion-exchange resins may be used quantitative purification of the streptomycin before applica of the analytical procedures.

CLINICAL USES OF STREPTOMYCIN

Streptomycin is indicated in the treatment of a variety o fections due to susceptible Gram-negative or acid-fast organ Although it is not a substitute for penicillin in the treatme infections due to Gram-positive, penicillin-sensitive microo isms, streptomycin does inhibit effectively the growth of Gram-positive bacteria. It may be employed successfully in treatment of certain cases of infection due to penicillin-resi strains of such pathogens. It should never be used in the ment of conditions caused by organisms found to be insuscep to therapeutic concentrations of streptomycin.

Among the infections in which streptomycin has been found
erapeutic value are certain urinary tract infections, tularemia,
enzal meningitis, bacteremia, and wound infections caused
treptomycin-sensitive organisms. It may often be used ad-
geously pre- or postoperatively for the prevention or control of
tions caused by susceptible pathogens. Intermittent intra-
ular injection is the preferred method of parenteral ad-
stration, although solutions of the drug may be also injected
utaneously in selected cases. When indicated, streptomycin
be given intraspinally, locally, or by inhalation. Since negligi-
mounts of streptomycin are absorbed from the gastro-intestinal
., oral administration is ineffective for the treatment of systemic
tions.

Dosage varies greatly with patient, infection, and route of
inistration. The rapidity with which many microorganisms
lop resistance to streptomycin is a factor which has indicated
tion of rather large initial doses. Dosage usually ranges from
to 4 g per day parenterally, additional amounts being given
uch other routes as may be indicated in the individual case.

In addition to its application in treatment of human diseases,
tomycin is now widely used in veterinary medicine. With large-
me production, the cost of the antibiotic is no longer a signifi-
factor in limiting its use.

It is now recognized that streptomycin is a valuable and, in
cases, an essential therapeutic adjunct in certain types and
s of tuberculosis, although it is not a substitute for such well-
lished methods of treatment as bed rest, special diet, and
uate surgical procedures. The proper combination of thera-
ic measures to be employed in the individual case must be
choice of the physician in charge.

Streptomycin therapy is considered mandatory in acute hem-
enous miliary tuberculosis and tuberculous meningitis, and
se is strongly indicated in tuberculous pneumonia. Strepto-
n is effective also in the treatment of selected cases of tuber-
is of the respiratory, genito-urinary, and gastro-intestinal
s. Certain types of pulmonary tuberculosis are greatly im-
ed by the proper employment of streptomycin in conjunc-
with traditional therapeutic methods. In many instances,
tomatic improvement is striking and x-ray findings of favor-
import are noted. However, it is by no means advisable to

employ streptomycin in all cases of pulmonary tuberculosis each instance, the physician must exercise critical clinical j ment to determine the thereapeutic regimen, with or wit antibiotic therapy, best suited to the individual case.[2,8,19]

Streptomycin is capable of producing side reactions of v ing severity. Most of those occurring early in the course of t ment are transient and not of a serious nature. However, se toxic reactions may occur in individuals who have become hy sensitive to streptomycin. Although antihistamine drugs or sensitization" procedures may be effective in such cases, in ce instances, further administration of streptomycin or contact it may have to be avoided.

When administered in comparatively high dosage over relatively long periods required for treatment of various of tuberculosis, certain neurotoxic reactions frequently becom important consideration. Interference with vestibular tion (impairment of the sense of balance) is most common has appeared in 96% of patients in a large series of cases. a dosage of 1.8 to 2.0 g per day, it usually appeared in the f week of treatment. Complete or partial functional recover various compensation mechanisms occurs in most cases, even continued treatment. Impairment of hearing may also occur this is relatively rare. Only 1% of a large series of cases sh sufficient toxicity of this type to make cessation of treatment able. If streptomycin therapy is continued after impairme hearing becomes evident, the disability may be expected to gress and become permanent. However, if administration o drug is discontinued as soon as signs or symptoms of such are noted, complete recovery may be expected in most cases.

Early clinical studies indicate that dihydrostreptomyc probably as effective as the parent drug, streptomycin, in treatment of certain types of tuberculosis and that it exhibit nificantly less neurotoxicity.

Hydroxystreptomycin has been tested for ototoxic effe cats. It had a neurotoxic effect greater than that of eith hydrostreptomycin or streptomycin and caused a severe he loss after very few doses.

Streptomycin is of great effectiveness in clinical medici a therapeutic agent. Therefore, it is widely used despite its comings. The two most serious drawbacks to its effective us the ease which microorganisms develop resistance to strepto he inherent neurotoxicity of the molecule to the animal body prolonged administration. With the development of dihy-reptomycin and the adoption of streptomycin dosage regimens ving relatively low daily doses for limited periods of therapy, problem of neurotoxicity has been greatly reduced. The de-ment of bacterial resistance to streptomycin and dihydro-tomycin therapy is a serious problem clinically. However, it ound in recent years that streptomycin or dihydrostreptomycin njunction with *p*-aminosalicylic acid or isoniazid (isonicotinic hydrazide) was generally more effective in tuberculosis chemo-py than streptomycin used alone.[32a] There is clear evidence *p*-aminosalicylic acid inhibits the development of resistance of *uberculosis* to streptomycin.

Streptomycin has also found application in the treatment of rial diseases of plants.[21b] Streptomycin is absorbed and stored e plant leaf. Since it is not removed by rainfall, the antibiotic in the leaf acts as a reservoir of protective agent effective against tion. It is especially valuable in bacterial diseases of valuable ts, such as fire blight of pears and walnut blight. It is also rted effective against bacterial wilt of chrysanthemum and blight of beans. Much research is needed before the final place reptomycin in plant-disease control is established. At high con-ations, streptomycin causes chlorosis and, at somewhat lower entrations, inhibits root development. However, the last-ioned toxic effect can be overcome by manganese salts.[32a]

The manufacture of streptomycin is one of the important fer-ation operations in the United States and abroad. It is useful inical medicine and new and broader applications are being d continuously. Further improvements in production, increased ledge of its activity, and wider applications may be anticipated the extensive research which is still in progress on streptomycin.

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CHAPTER 9

THE BROAD-SPECTRUM, POLYPEPTIDE, AND OTHER ANTIBIOTICS

William H. Peterson and Mary S. Peterson

INTRODUCTION

Under this title are included antibiotics of microbiologica origin other than penicillin, streptomycin and dihydrostrepto mycin, since these products are dealt with by other authors i separate chapters. Actidione (cycloheximide), Aureomyci (chlorotetracycline), bacitracin, Chloromycetin (chloramphen col), Erythrocin (erythromycin), fumagillin, Ilotycin (erythrom cin), Magnamycin (carbomycin), neomycin, polymyxin, Terram cin (oxytetracycline), tyrothricin, tetracycline, and viomycin mak up the list of antibiotics that are now (February, 1953) mark products. Many of these are better known by their tradema names (first letters in capitals) than by their scientific names. Th system of double names is confusing and troublesome. It is esp cially so when trade names that have become well-established i scientific literature, such as Aureomycin and Terramycin, are wit drawn from such use and replaced by a chemical designation.

The bulk of the antibiotics industry is made up of penicilli streptomycin and the so-called "broad spectrum" antibiotics, Aure mycin, Chloromycetin, and Terramycin. The latter, though scarce five years old, have established themselves as leaders from a ustrial, economic, and clinical point of view. In June, 1952, lch[287a] reported that the production of "broad spectrum" anti tics amounted to 24 tons per month as compared to 30 tons for icillin and 24 tons for streptomycin. In tonnage, the two older ibiotics exceed the "broad spectrum" antibiotics, but in mar value the latter appear to equal or outrank the former. The rket price of the "broad spectrum" antibiotics in capsule form ges from $2.13 per gram for Aureomycin to $1.20 per gram for loromycetin. These prices are subject to trade discounts, but en allowing for these and for the unequal production of the ree antibiotics,[286a] it seems probable that the returns to the ducers of the "broad spectrum" antibiotics is well over 200 llion dollars annually. The returns from penicillin and strepto cin, when compounded into tablets, injectable solutions, sus nsions, and many other dosage forms, is probably of the same der of magnitude. If the income from the minor antibiotics d from the sale of antibiotics as supplements to animal feeds added to these sums, a total of about 500 million dollars yearly ms a reasonable figure for the size of this huge and expanding dustry.

Besides the commercial antibiotics, there are several others at either have not been tested sufficiently to establish their use ness or for which there still appear some commercial possi ities under certain circumstances even though they are too xic for widespread use. Some of these are new types of com unds and others contain new examples of old types, e.g., amino ds. Although they may be too toxic for human therapy, some y prove useful in the treatment of animal diseases or in com ting insect pests and plant diseases. Many manufacturers have e or more antibiotics in various stages of development but nsideration of these will have to wait until information about em has been released. It is probably a reasonable estimate that ,000 new isolates are tested yearly by the producing companies. any new, interesting and, hopefully, useful compounds should me out of this vast number of cultures.

Many antibiotics, uncovered in this search but only partly rified, have been abandoned by producers because toxicity or er undesirable features make them too unpromising for fur r work. If these could be studied and their chemical nature ermined (for example, by university laboratories) it would

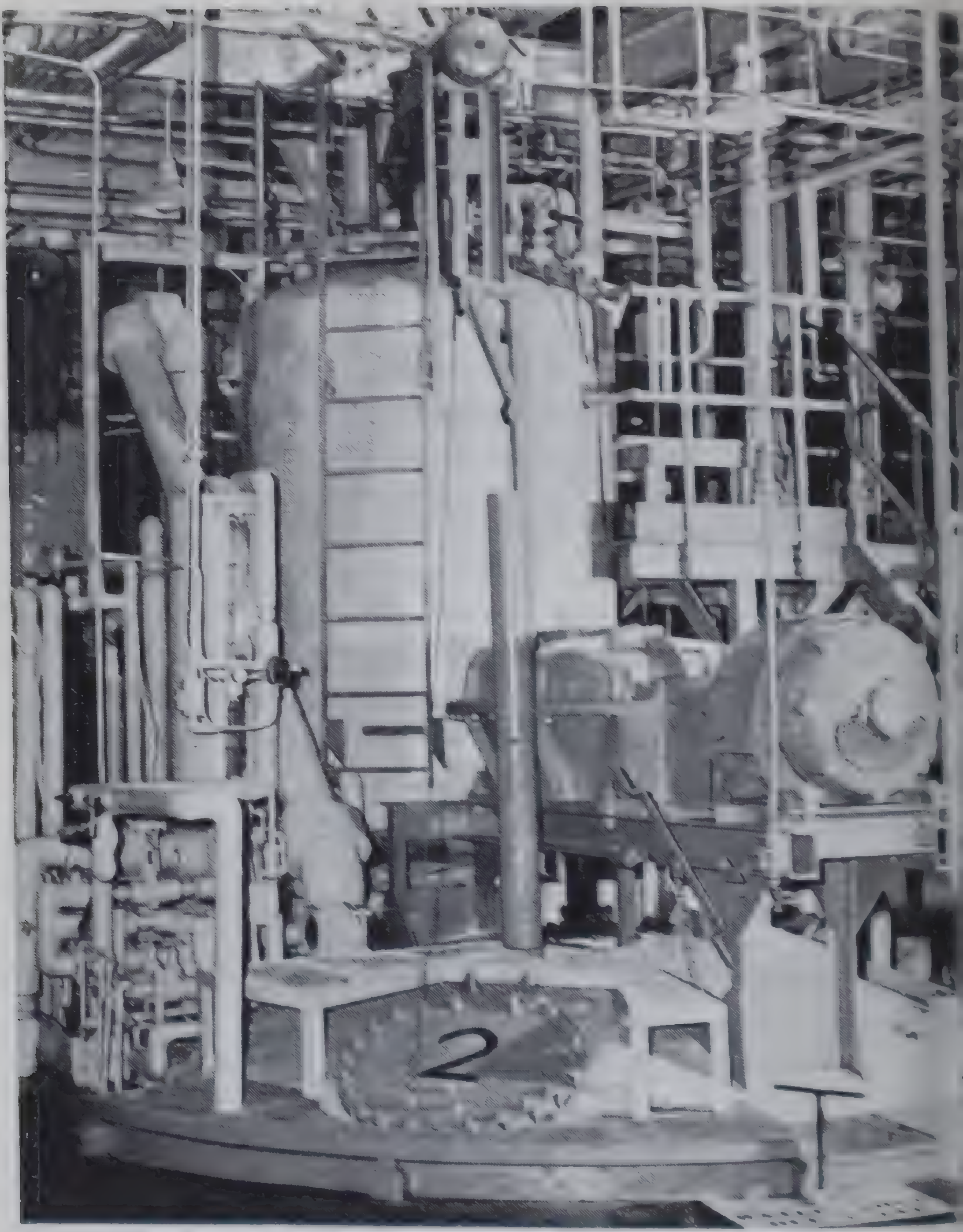


FIGURE 20A. *Seed and Fermentation Tanks Used in the Producti*
of Antibiotics (Courtesy—Lederle Laboratories D
sion, American Cyanamid Company)

provide a body of knowledge that might be very valuable in
riving at some rational explanation for antibiotic activity.

In addition to the antibiotics discussed in some detail in
chapter, there is a second group that is listed in a table giving
most important facts about them. There are probably close

dred others for which the data are too scanty to warrant in- ion in this table. For a more complete list of antibiotics, uding those of plant origin, reference is made to recent ks,[19a,105,151,211] and reviews.[18,26,57,130,153,198,204a,299,305a]

In the discussion that follows, emphasis has been placed on production, isolation, and chemical nature of the antibiotic ier than on its activity and toxicity. However, some mention he therapeutic value could not be omitted because clinical use mately determines the commercial production of an anti- tic. The antibiotics are discussed in alphabetical order rather n in the order of their industrial importance. The latter ar- gement would not be possible, since no figures are available ere there is only one producer and the possible importance of noncommercial products is still uncertain.

In writing this report, the authors are regretfully aware that ch of the information will be outdated before these pages ap- r in print. The printed page is always out of date, but is con- uously so in the rapidly developing field of antibiotics.

COMMERCIAL ANTIBIOTICS

Actidione (Cycloheximide)

During the commercial production of streptomycin by The john Company, three of their research workers[295] discovered t the beers used for that production contained also another ibiotic which they named Actidione. This is the trade name l cycloheximide is the chemical name.

DUCTION

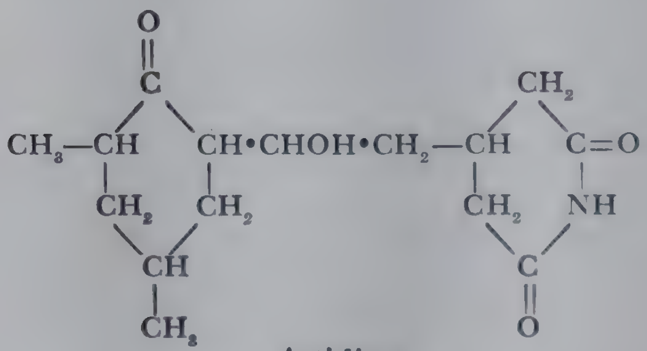
Whether *Streptomyces griseus* produces greater quantities of ptomycin or of Actidione seems to depend on the medium used its growth. In a glucose (Cerelose)-salts medium supple- nted by dried brewers yeast, high yields of streptomycin were ained, but little Actidione. In another medium supplemented soybean meal and Curbay BG (the dried solids in the spent rs from the butanol fermentation of molasses; a U. S. Industrial emicals, Inc., product) instead of yeast, the yield of Actidione higher than that of streptomycin. Tests showed that the rbay BG was the constituent of the medium that stimulated duction of Actidione. Since Curbay BG itself is a very complex substance, it would be interesting to know which of its ingredients promotes the formation of Actidione.

Shaken flasks were used for the fermentation at 24°C and assay for Actidione was made by the paper-disk method. Mutations (x-ray) that destroy the capacity of the organism to produce one of these antibiotics usually destroy the ability to produce the other also, but this is not invariably the case.[294]

Yields of Actidione average 80 to 100 mg per l, but in a few cases 200 to 250 mg per liter has been obtained. A crude product, 30 to 60% pure, was obtained from the beer by extraction with chloroform, adsorption on activated carbon, elution with acetone, and removal of acetone by distillation. Chromatography proved most convenient for isloation of the crystalline compound.[108]

Chemical Properties

An empirical formula, $C_{27}H_{42}N_2O_7$, was reported for Actidione by Leach, Ford, and Whiffen,[163] but further study[108] showed the formula to be $C_{15}H_{23}NO_4$. More detailed investigation[158,159] of its chemical nature, by chemical degradation and transformations and physical studies, established the structural formula:



Actidione

From this formula, it is apparent that there is only one ketoni group instead of two, so that the name Actidione is actually a misnomer.

Crystalline Actidione has a melting point of 115° to 117°C Its specific rotation is—3.0° in methanol and +6.8° in water. I is very soluble in the common organic solvents, except the sat urated hydrocarbons. Its solubility in water is 2.1 g per 100 ml a 2°C.

Activity and Toxicity

Actidione has little activity against bacteria, but is very poten against a number of yeasts and some other fungi. It inhibits a fungal pathogen, *Cryptococcus neoformans,* and may prove o value in the treatment of the serious human infections caused by this organism.[127]

Its most promising use seems to be in combating fungal plant diseases, such as those infesting golf greens and other turfs.[10a,267] It is also useful in the laboratory as an aid to the detection and isolation of bacteria from samples contaminated with molds and yeasts. Addition of Actidione to the agar medium inhibits the growth of the fungi, but allows the bacteria to develop on the plates.[118a,122b] The toxicity of Actidione for animals varies greatly with the species, e.g., from $LD_{50} = 2.5$ mg per kg for rats intravenously to 150 mg per kg for mice by the same route. Its toxicity to plants likewise varies with the species.

Aureomycin (Chlorotetracycline)

SOURCE

Aureomycin is produced by the Lederle Laboratories, Inc., a subdivision of American Cyanamid Co. Aureomycin is the trademark name and chlorotetracycline is the generic name. Tetracycline is the basic-structure name agreed on by the producers[247] of Aureomycin and Terramycin (oxytetracycline).

This antibiotic is produced by a streptomyces which was isolated from a soil sample collected from a timothy field in Missouri. It is described[49,81,82] as a new species and named *Streptomyces aureofaciens* because on agar "at a certain stage in the growth of the colony . . . there is typically the production of a golden yellow pigment in the moist hygrophorus substrate mycelium." Several different strains of the organism have been obtained and one of these, A-377, is described in a patent[82] covering the production of the antibiotic. A transfer of *S. aureofaciens*, strain A-377, has been deposited in the collection of the Northen Regional Research Laboratory, Peoria, Illinois, under the number NRRL-2209. The antibiotic is "faintly golden yellow" in color, which feature has given rise to the name Aureomycin.

PRODUCTION

The original patent[82] states that *S. aureofaciens* can utilize many different kinds of carbohydrate and nitrogenous substances for its growth. As an example of flask fermentations the following is given: 100 ml of medium containing 1 to 3% sucrose, 1 to 2% corn-steep liquor, 0.5% $(NH_4)_2HPO_4$, 1.5% KH_2PO_4, 0.2% $MgSO_4$

•$7H_2O$, and traces of Mn, Cu, Zn, adjusted to pH 6.2, are placed in a 500-ml Erlenmeyer flask, sterilized, inoculated, and then shaken continuously at 28°C for 24 to 36 hours. From 500 to 2,000 Aureomycin units (usual range 1,000 to 1,500) are produced. A unit of Aureomycin is defined as the quantity of the antibiotic required to inhibit the growth of *Staphylococcus aureus* in 10 ml of a nutrient broth. Judging from later reports on the weight of pure Aureomycin required to inhibit assay strains of *S. aureus,* a unit is probably in the neighborhood of 0.5 μg per ml. The yields of Aureomycin given in the patent, if calculated to a weight basis, are in the order o 500 to 750 mg per liter. Judging from the improvement of penicillin cultures, the current yields could be much greater than the 500 to 750 mg figures. Van Dyck and De Somer[265a] reported yields of 1,30 mg per liter in shaken flasks for an improved culture and tan fermentations should be higher.

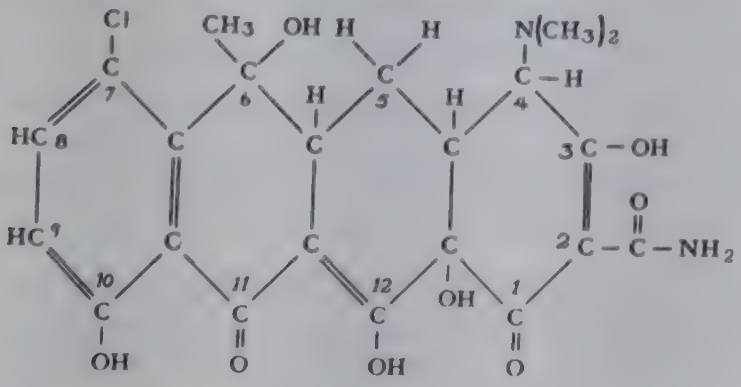
In deep-tank fermentations, the medium is somewhat mor dilute and also contains 0.1% $CaCO_3$. During fermentation, th medium is kept at 26° to 28°C and aerated at the rate of 0.5 to 1.5 of air per liter of medium for 24 to 48 hours. The pH of the mediu may decrease to 4.5 during fermentation, but in a later patent,[19] the drop in pH is said to be undesirable and is avoided by using u to 1% $CaCO_3$ to maintain the pH between 6.4 and 7. This paten emphasizes further the importance of calcium carbonate as an ai to increased Aureomycin production, because the calcium ion r acts with Aureomycin, as it is formed, to give a precipitate and thu promote the production of more Aureomycin. The yield reporte 396 μg per ml, seems distinctly low. The patents give no addition information as to the sequence of chemical changes in the mediu and the relation of these changes to Aureomycin formation.

A recent biochemical study of the Aureomycin fermentatio has been made by Van Dyck and De Somer.[265a] In this study, cu tures selected from spores that had been exposed to ultraviolet ligh were employed. The spores were plated, colonies were examined f morphological characteristics (color, sporulation, and colony form and those selected were then tested for Aureomycin productio From 1,457 colonies, 9 strains were obtained that gave arou 1,300 μg Aureomycin per ml as compared with about 800 for th parent culture. The fermentations were done with 50 ml mediu in Erlenmeyer flasks on a rotary shaker at 250 rpm, for about hours at 24° to 30°C. The medium was essentially that given ne Aureomycin patent, but with added peanut meal and molasses. The chemical changes in the medium were as follows: The sucrose ontent fell rapidly from 3% at the start to about 0.1% after 0 hours. Formation of Aureomycin became marked after 15 hours nd continued uniformly for about 35 hours and then leveled off. During this time, about 900 μg per ml of Aureomycin was formed r about 25 μg per ml per hr. This rate compares favorably with hat of other antibiotic fermentations. With the exhaustion of the ucrose supply, there was little additional formation of Aureomycin. The pH of the medium remained fairly constant at 6.4 to 6.6 during he first 50 hours and then rose rapidly to 7.1 at 70 hours. The ise in pH and cessation of antibiotic production with exhaus-ion of sugar follow the familiar pattern observed in penicillin ermentations.

Pidacks and Starbird[209a] describe the recovery of Aureomycin rom the broth by chromatographing on a silicate column with an cidulated solvent, collecting the fraction that is yellow under ultra-iolet light, concentrating and extracting this with butanol, and inally precipitating the Aureomycin as the hydrochloride with ther.

A later patent[4d] gives a recovery procedure that seems better dapted to large volumes of fermentation broth. The Aureomycin precipitated with lime at pH 8.8 is then extracted from the filter ake with sulfuric acid at pH 1.35. The filtrate is evaporated in acuum at 30°C to about one fourth its volume, a small quan-ity of butanol is added, and the solution nearly saturated with odium chloride. On standing overnight at 2° to 5°C, the Aureomycin crystallizes as the hydrochloride of about 97% purity.

CHEMICAL NATURE AND PROPERTIES[40,82,282]



Unraveling the structures of Aureomycin and Terramycin is one of the recent brilliant achievements in organic chemistry. This successful work resulted from the efforts of two large groups of chemists working separately on the respective antibiotics. To date seven papers have appeared on Aureomycin and eight on Terramycin. Only the last paper in each series will be cited, as reference to one or more of the preceding communications is made in the last paper of each series.

The structures of Aureomycin and Terramycin are very similar. Both contain a naphthacene nucleus and all but two of the modifying groups are alike in both antibiotics. Compared with Aureomycin, Terramycin has a hydroxy group at position 5 instead of a hydrogen and a hydrogen at position 7 instead of a chlorine atom. In effect, it has a hydroxyl instead of a chlorine but, oddly enough, the two occupy different places in the naphthacene skeleton. The other hydroxyl, amide, carbonyl, and methyl groups are the same in number and position in both antibiotics. The basic structure common to both Aureomycin and Terramycin is called tetracycline and thus Aureomycin is named chlorotetracycline and Terramycin is designated oxytetracycline.

Aureomycin is a weak base that contains both nitrogen and nonionic chlorine. The free base has a melting point of 168° to 169°C, a levo rotation $[\alpha]_{\mathrm{D}}^{23^\circ} = -275^\circ$ (in methanol), and a solubility in water of 0.5 to 0.6 mg per ml. It is much more soluble in dilute acid or alkali. Its solubility in organic solvents is reported as follows: very soluble in pyridine, Cellosolves, and dioxane slightly soluble in methanol, butanol, and ethyl acetate; insoluble in ether and petroleum ether. Aureomycin forms a crystalline hydrochloride containing one equivalent of hydrochloric acid pe mole of base. In 0.1 N hydrochloric acid, three absorption maxima occur at 230, 262.5, and 367.5 mμ. In sodium hydroxide, the peak are all shifted to longer wave lengths.

Systematic study has been reported of the stability of Aureomycin in connection with its assay and tests against bacteria.[79,212] Thes tests show that it is a rather unstable compound which is surprisin considering its effectiveness. Even in distilled water, about 50% o the activity was lost in 24 hours at 37°C. In phosphate buffer pH 7.0 or in the presence of peptone, blood serum, and many othe substances, deterioration was increased. Acidity (pH 2) and low temperature (4°C) increased the stability.

CTIVITY

Aureomycin is an unusually versatile antibiotic; it is active *n vitro* against numerous Gram-negative and Gram-positive baceria and appears particularly potent against the spore-forming erobes.[37c,202,212] Because it acts on many Gram-positive and Gram-egative bacteria as well as rickettsiae and certain viruses, it is alled a "broad spectrum" antibiotic. Chloromycetin and Terranycin also belong in this category. Although Aureomycin is not s effective as penicillin against infections caused by Gram-positive acteria, its use in such infections has given good results especially n cases that have become resistant to penicillin. Aureomycin apears to be very effective against rickettsial diseases, such as Rocky Mountain spotted fever, scrub typhus fever and typhus fever[98,303] nd less effective against Q fever.[166] Notable results have been obained in the treatment of psittacosis, lymphogranuloma venereum, ranuloma inguinale, and virus pneumonia.[303,306] Somewhat romising results have been reported against brucellosis,[75,123a,242,303] mebiasis,[182b] and whooping cough.[24,128e] Aureomycin is apparently neffective against tuberculosis and also against infections caused by roteus- and pseudomonas-type bacteria.

Microorganisms that develop resistance to Aureomycin are also esistant to Terramycin and vice versa.[203b] This result is to be exected since both antibiotics have the same basic chemical structure. ome of these resistant organisms are also resistant to Chloromyetin.[111b] This effect has been explained on the assumption that all hree antibiotics act on microorganisms in the same way. Prolonged ngestion of Aureomycin, Chloromycetin, and Terramycin may lead o marked changes in the fecal flora and to the development of econdary fungal infections in the mouth and gastro-intestinal ract.[201a,203b] This infection is called moniliasis as the organisms ausing it are classed as monilia (old name for candida). An exmple is *Candida albicans.* Such organisms are not inhibited by the road-spectrum antibiotics. Therefore, when the normal inhabitants f the intestinal tract (the colon-aerogenes bacteria) are suppressed, he monilia have a good opportunity to develop.

OXICITY

The intravenous LD_{50} for mice is 134 mg per kg.[128] In clinical ork, it is usually given by mouth and in some cases nausea and diarrhea have been experienced, but no consistently serious effects have been observed.[98,303]

Bacitracin

Source

Bacitracin is produced by a strain of *Bacillus licheniformis,*[184] formerly classified as *Bacillus subtilis,* strain Tracy. It was isolated from infected tissue removed at the time of surgical operation from the fractured tibia of a 7-year-old child, named Margaret Tracy. The first announcement of the isolation of the organism and naming of the antibiotic was made in 1945 by Johnson, Anker, and Meleney.[147] Since that time, many papers have been published regarding bacitracin and in 1948, it was produced semicommercially and put on the market by Commercial Solvents Corporation.[10] The new bacitracin plant of the company began operating late in 1951.[143a]

A unit of bacitracin is defined as the amount of the antibiotic which when dissolved in 1024 ml of beef infusion broth completely inhibits the growth of a group A hemolytic streptococcus under specified conditions of inoculum and incubation.

Bacitracin, as commercially produced, is a light-gray, hygroscopic powder of bitter taste. Its purity is approximately 45 units per mg.[143a] The purest preparation of bacitracin obtained to date had a potency of 66 units per mg.[70] One unit is, therefore, equivalent to about 15 μg. This weight per unit is very large in compariso with penicillin (one unit equals 0.6 μg) and streptomycin (one uni equals 1.0 μg). Comparison of antibiotics on the basis of units i meaningless and even misleading. An antibiotic similar to bacitracin was obtained by a group of English workers[197] from a differen strain of *B. licheniformis.* Originally this antibiotic was calle ayfivin, but after careful purification and comparison with bacitracin, it was decided that several of the constituent polypeptides i bacitracin and ayfivin were identical and the name ayfivin has therefore, been abandoned.

Production

Various media have been used in flask and pilot-plant fermentations.[8,175] One of the most important constituents for high yield is a plant protein, such as soybean or peanut meal. Yields up t

00 units per ml, or about 1.5 mg per ml on a weight basis, have een obtained on such media. This yield is in about the same range s that for penicillin and Aureomycin.

Production on a plant scale has recently been reported[143a] in new plant built and operated by the Commercial Solvents Corpo-ation. The layout of the plant and a description of its operation rom stock culture to finished product are given. A summary of the rocess will be listed here, but for details, the original report should e consulted. A flow sheet is shown in Figure 21.

The medium used in plant production differs somewhat from hat used in the laboratory. Because of the greater aeration in anks, the bacteria grow more rapidly and bring about more ex-ensive deamination of proteins with accompanying rise in the pH f the medium. To offset this effect, the medium is balanced with arbohydrate. When metabolized, this reduces deamination and lso yields acids which counteract the alkaline products resulting rom the metabolism of proteins. Dextrose, sucrose, and starch are uitable carbohydrates, but maltose gives low bacitracin titers. From suitable medium, Lee[165a] reports titers of more than 100 units per nl in 36 to 48 hours. This is a shorter period than that required or mold and streptomyces fermentations.

The fermentation process starts with spores of *B. licheniformis* (*B. subtilis*) kept in sterile soil. These are inoculated into a 4-l flask f peptone or tryptone broth. The flask is incubated at 37°C and haken continuously for 18 to 24 hours. The contents of the flask re then used to inoculate media in a 150-gal stainless-steel tank. he culture is aerated for 6 hours and is then blown by sterile air nto a 1,200-gal seed tank containing previously cooked mash ooled to 37°C. The seed tank is aerated and when proper growth as been attained, the seed is used to inoculate a 24,000-gal fer-nentor. During fermentation, sterile air is forced through the nash, but apparently, simultaneous agitation (used in most anti-iotic fermentations) is not employed. As the fermentation pro-eeds, the pH of the medium rises gradually. Formation of acitracin begins about midway in the period, presumably at about 8 hours, and increases rapidly to the end of the fermentation. nfortunately, little information is given on the chemical changes hat take place during the fermentation. In justice to the writers, should be said that this criticism applies not only to the bacitra-in fermentation, but also to those for Aureomycin, Terramycin,

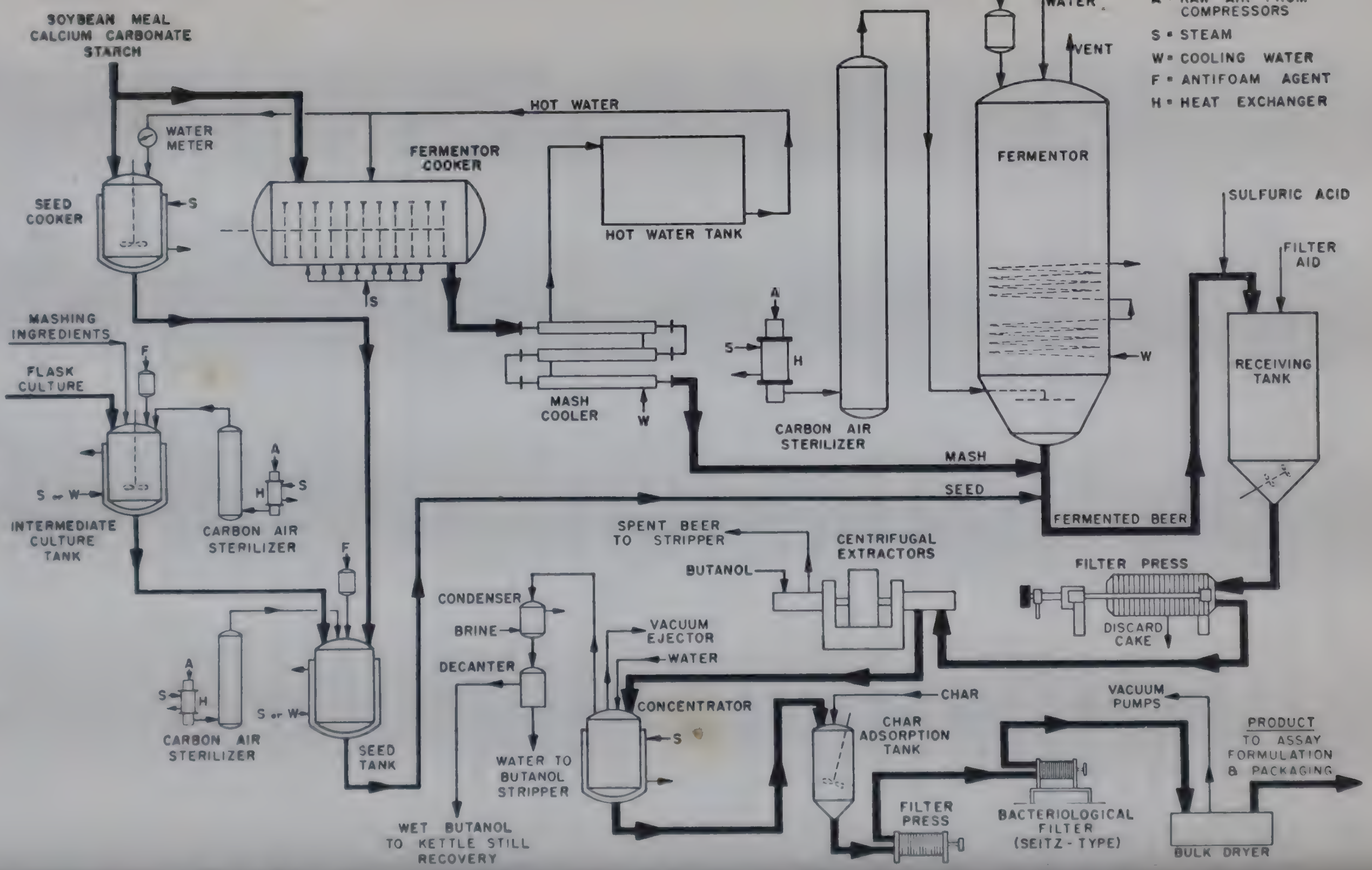

A = RAW AIR FROM COMPRESSORS
S = STEAM
W = COOLING WATER
F = ANTIFOAM AGENT
H = HEAT EXCHANGER
SOYBEAN MEAL
CALCIUM CARBONATE
STARCH
WATER METER
SEED COOKER
S
HOT WATER
FERMENTOR COOKER
S
HOT WATER TANK
MASHING INGREDIENTS
FLASK CULTURE
F
INTERMEDIATE CULTURE TANK
S or W
CARBON AIR STERILIZER
A
S
H
MASH COOLER
W
CARBON AIR STERILIZER
A
S
H
WATER
VENT
FERMENTOR
W
MASH
SEED
FERMENTED BEER
SULFURIC ACID
FILTER AID
RECEIVING TANK
FILTER PRESS
DISCARD CAKE
F
CARBON AIR STERILIZER
A
S
H
S or W
SEED TANK
SPENT BEER TO STRIPPER
BUTANOL
CENTRIFUGAL EXTRACTORS
CONDENSER
BRINE
DECANTER
WATER TO BUTANOL STRIPPER
WET BUTANOL TO KETTLE STILL RECOVERY
VACUUM EJECTOR
WATER
CONCENTRATOR
S
CHAR
CHAR ADSORPTION TANK
FILTER PRESS
BACTERIOLOGICAL FILTER (SEITZ-TYPE)
VACUUM PUMPS
BULK DRYER
PRODUCT
TO ASSAY FORMULATION & PACKAGING

FIGURE 81.—*Flow Diagram for Production of Bacitracin.* [Reprinted by permission from a paper

d other commercial antibiotics. Such information, the reviewers
lieve, could be given without disclosing the exact composition of
e medium and would add greatly to our knowledge of the metab-
ism of these important industrial microorganisms.

Bacitracin is recovered from the fermented medium by acidi-
ing the broth, filtering it to remove cells and other solids, and
tracting the filtrate with butanol. The solvent is removed in a
cuum below 28°C, with simultaneous addition of water to keep
e bacitracin in solution. The aqueous concentrate is further
irified with activated charcoal to remove color and other im-
irities. It is then passed through a Seitz filter to remove any chance
cteria, and finally it is freeze-dried. The light-gray, hygroscopic
owder thus obtained is the product of commerce and contains
oout 45 units of bacitracin per mg. If 60 units per mg is taken as
ie potency of pure bacitracin, the product has a purity of
oout 75%.

HEMICAL NATURE AND PROPERTIES[8,20,70,70a,70b,143a,175,197,210a]

Bacitracin appears to be a mixture of polypeptides, contains
oout 14 to 15% nitrogen, and gives positive biuret, Hopkins-Cole,
id xanthoproteic tests, but negative Millon and Sakaguchi re-
ctions. It is precipitated from solution by many of the common
rotein reagents, such as heavy metals, trichloroacetic acid, phos-
hotungstic acid, and even by saturation with sodium chloride.

The commercial product appears to consist of several anti-
acterial polypeptides designated bacitracin A, B, C, D, E, and F.
he A component makes up the main part of the product with
arying amounts of minor components. Three of these proved
lentical with components found in another antibiotic, called
fivin, but this name is now abandoned in favor of bacitracin.

Commercial bacitracin has been highly purified by counter-
irrent distribution and the A component obtained free from other
mponents. It has been hydrolyzed with acid and the amino acids
etermined by ion-exchange-column chromatography. One mole
ch of L-cysteine, L-histidine, L-leucine, L-lysine, D-phenylalanine,
glutamic acid and D-ornithine and 2 moles each of DL-aspartic acid
id L-isoleucine were found. However, only 90.7% of the total nitro-
n and 87.4% of the weight of peptide taken were recovered in the
nino acids. The same amino acid composition was reported for
citracin purified by carrier-displacement chromatography. The

molecular weight of bacitracin A obtained by direct determination, 1411 to 1450, is in good agreement with that calculated from the amino acid content.

Bacitracin is soluble in water, methyl and ethyl alcohols, glacial acetic acid, and formamide; slightly soluble in *n*-propyl and other alcohols; and insoluble in ether, chloroform, and several other organic solvents.

Bacitracin is adsorbed from aqueous solution; for example, by charcoal, Super Filtrol, but not by activated alumina. The ultraviolet absorption curve shows a small peak at 253 mμ.

Bacitracin is a very stable antibiotic. In water at 100°C, 85% of the activity remains after 30 minutes and at refrigerator temperatures, full activity is retained for about 3 weeks. It is most stable a pH 4 to 5. At pH 1.6 at 37°C, about 60% of the activity is lost i 24 hours. It is not inactivated by pepsin or trypsin, but seems somewhat labile to papain. It is inactivated by ultraviolet light.

ACTIVITY AND TOXICITY[10,17,76,89,90,145b,147,175,183,235]

Bacitracin resembles penicillin in its bacterial spectrum; i is most active against Gram-positive bacteria. However, man organisms in this group that are resistant to penicillin are sensitiv to bacitracin. When used together, the two antibiotics appear t be synergistic, but in other combinations an antagonistic effect ha been noted.

The LD$_{50}$ for mice was 240 mg per kg for preparations havin an activity of 35 units per mg where this was administered intraperitoneally. It brings about serious kidney damage in mice and mo patients given bacitracin show more or less albumin in the urin For this reason it should not be injected and its use is general restricted to topical application or to local infiltration into wounc and pus-forming lesions. It is reported to have given good resul in many surgical cases and in the treatment of various skin disease

Chloromycetin (Chloramphenicol)

SOURCE

Chloromycetin is the trade name and chloramphenicol is th generic name given to this antibiotic by the producer, Parke, Dav and Co.

The actinomycete which produces Chloromycetin is a ne cies[91,92] named *Streptomyces venezuelae* and since the compound ntains chlorine, it is quite evident how the word Chloromycetin s devised. Three cultures from widely separated geographical gions have been isolated and found to produce Chloromycetin. ne of these[91] came from a soil sample collected near Caracas, nezuela, another[58,122] from a compost soil on the farm of the inois Agricultural Experiment Station at Urbana, and a third om Japan.[264] According to Umezawa, Tazaki, and Fukuyama[263] hloromycetin-producing streptomyces are widely distributed in panese soils and cultures can be readily isolated by adding 0.025 0.05 mg of Chloromycetin per ml to the agar plate before inulating with soil samples. The cultures from Venezuela and linois have been carefully compared and appear to be identical as dged by morphology, nutrition, antibiotic production, and other sts.

RODUCTION

In preliminary trials,[92] the Venezuela culture gave slightly gher yields than the Illinois culture and was the organism used industrial production.[226] In laboratory fermentations,[240] the est yield (169 μg per ml) was obtained on a medium containing 0% glycerol, 0.5% B-Y fermentation solubles (Commercial Solnts Corp.), 0.5% tryptone (Difco), and 0.5% NaCl. Glycerol ems to be superior to sugars as a carbohydrate source and animal oducts, *e.g.*, hog stomach residue, and hydrolyzed soybean protein e excellent sources of nitrogen. In 2,000-gal vat fermentations, μg per ml has been reported.[93] The Japanese investigators, mezawa and associates,[260,261,367] have published a number of papers aling with the production of high-yielding mutants by treatment the parent culture with ultraviolet light and improvement of the edium by addition of various nutrients. Their best culture, 33-115, which is also nearly free of pigment, gave yields of 440 μg r ml in flask fermentations and 300 μg per ml in 150-l batches. ne medium contained 2% starch, 0.5% beef extract, 0.5% peptone, d 1% NaCl. A study of the effect of amino acids on Chloromycetin oduction showed that phenylalanine, tyrosine, and methionine ed singly greatly increased the yield. The collective effect of these nino acids was even greater. For example, 480 μg per ml was tained with all three amino acids when 0.01% of each was added the previously described natural medium. Several possible pre-

cursors of Chloromycetin were tested, but only dichloroacetic ac
gave any response and the results with this compound we
irregular.

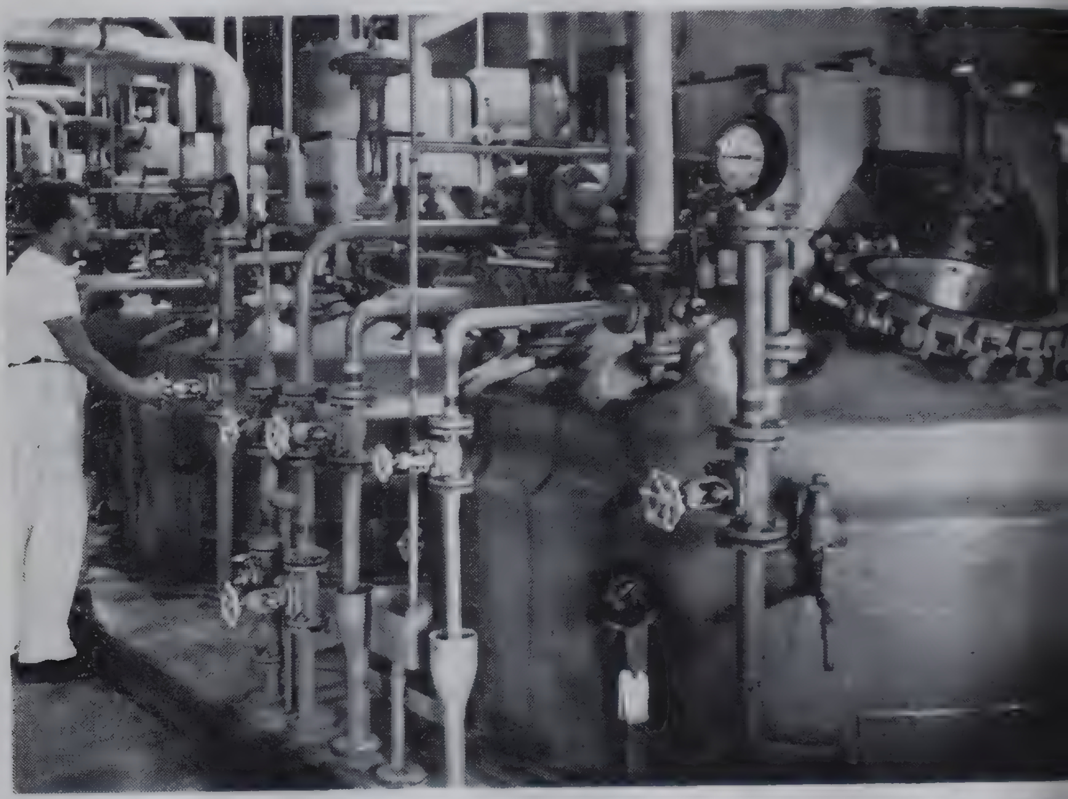


FIGURE 21a. *Fermentors Used for Production of Antibio*
(Courtesy—Parke, Davis & Company)

Data on the chemical changes brought about in the medi
have been reported for flask fermentations.[201] Production of
celium and Chloromycetin run parallel and reach a maximum
mg and 74 μg per ml respectively in about 3 days. The parallel
between mycelium and Chloromycetin production is quite diffe
from the relationships found in penicillin and streptomycin
mentations, in which antibiotic production is not marked until a
mycelial growth is well developed. In the Chloromycetin ferme
tion, glycerol was used up and ammonia nitrogen was at its lo
level after 3 days. Autolysis then set in as was evidenced by decr
in weight of mycelium and increase in ammonia nitrogen. T
were no marked changes in the pH of the medium. In a ferme
tion started at 7.4, the pH of the medium decreased to 7.0 and
rose slowly to 8.2 at the end. In an earlier paper,[210] the pH of
medium was given as about 6 or below throughout the fermenta

parently, Chloromycetin can be formed both in acid and slightly
line media. An interesting feature of the fermentation was the
pearance of an unknown reducing substance simultaneously with
rapid utilization of glycerol.[201] About 70 mg of reducing sub-
nce per 100 ml, calculated as glucose, accumulated during the
ond day of fermentation and disappeared by the end of the third
. During the second day, more than 500 mg of glycerol was used
100 ml.

A somewhat different sequence of events is described in a later
per by Legator and Gottlieb.[165b] They found that maximum
wth was reached about 48 hours before the peak yield of
loromycetin. An interesting feature of their work was the effect
biosynthesis of adding Chloromycetin to the medium. The same
el of Chloromycetin, about 100 μg per ml, was reached with or
hout additions. In other words, added Chloromycetin merely
vented the formation of equivalent amounts of Chloromycetin.
nfirmation of these unexpected results under more varied condi-
ns and with higher yielding strains would seem desirable and
rth while. For comparison, the authors made similar experiments
h the Terramycin producer, *S. rimosus,* but additions did not
vent the normal synthesis of Terramycin.

Chloromycetin can be obtained readily from the broth[21,22] by
ering off the mycelium and solids of the medium with the aid of
per Filtrol and Hyflo Super-Cel and extracting the antibiotic
m the filtrate at pH 8.5 to 9.0 with ethyl acetate. The extract is
centrated until it contains 20 mg of Chloromycetin per ml, a
rter volume of kerosene is added to keep impurities in solution,
l, after being washed with dilute acid and alkali, the extract is
ed over sodium sulfate. The solvent is partially removed by
uum distillation and, on standing at 5°C, Chloromycetin crystal-
s out. The crystals are purified by a second crystallization from
ylene dichloride and then melt at 149.2° to 150.2°C. Recovery,
n broth to final crystals, is about 70%. Second crops of crystals
e raised the recovery to over 80%.

Chloromycetin has been synthesized in several ways. An out-
of one of these syntheses is as follows: Condensation of
zaldehyde and nitroethanol is followed by reduction of the
densation product to the amino compound, C_6H_5·CHOH
INH$_2$·CH$_2$OH; acetylation of the amino and primary alcohol
ups gives C_6H_5CHOH·CH (NHAc)CH$_2$OAc; separation of the

DL-erythro form by crystallization, acetylation of the secondary a
cohol group, and nitration to $O_2N\cdot C_6H_4\cdot CH(O\cdot Ac)\cdot CH(NHA$
CH_2OAc are followed by hydrolysis, resolution of the base vi
D-camphorsulfonic acid to $O_2N\cdot C_6H_4\cdot CHOH\cdot CHNH_2\cdot CH_2OH$
and condensation with methyl dichloroacetate to give Chlorom
cetin, $O_2N\cdot C_6H_4\cdot CHOH\cdot CH(NH\cdot CO\cdot CHCl_2)CH_2OH$. For detai
of the many steps involved in the syntheses, the reader is referre
to the original papers[66,169] and patents.[72]

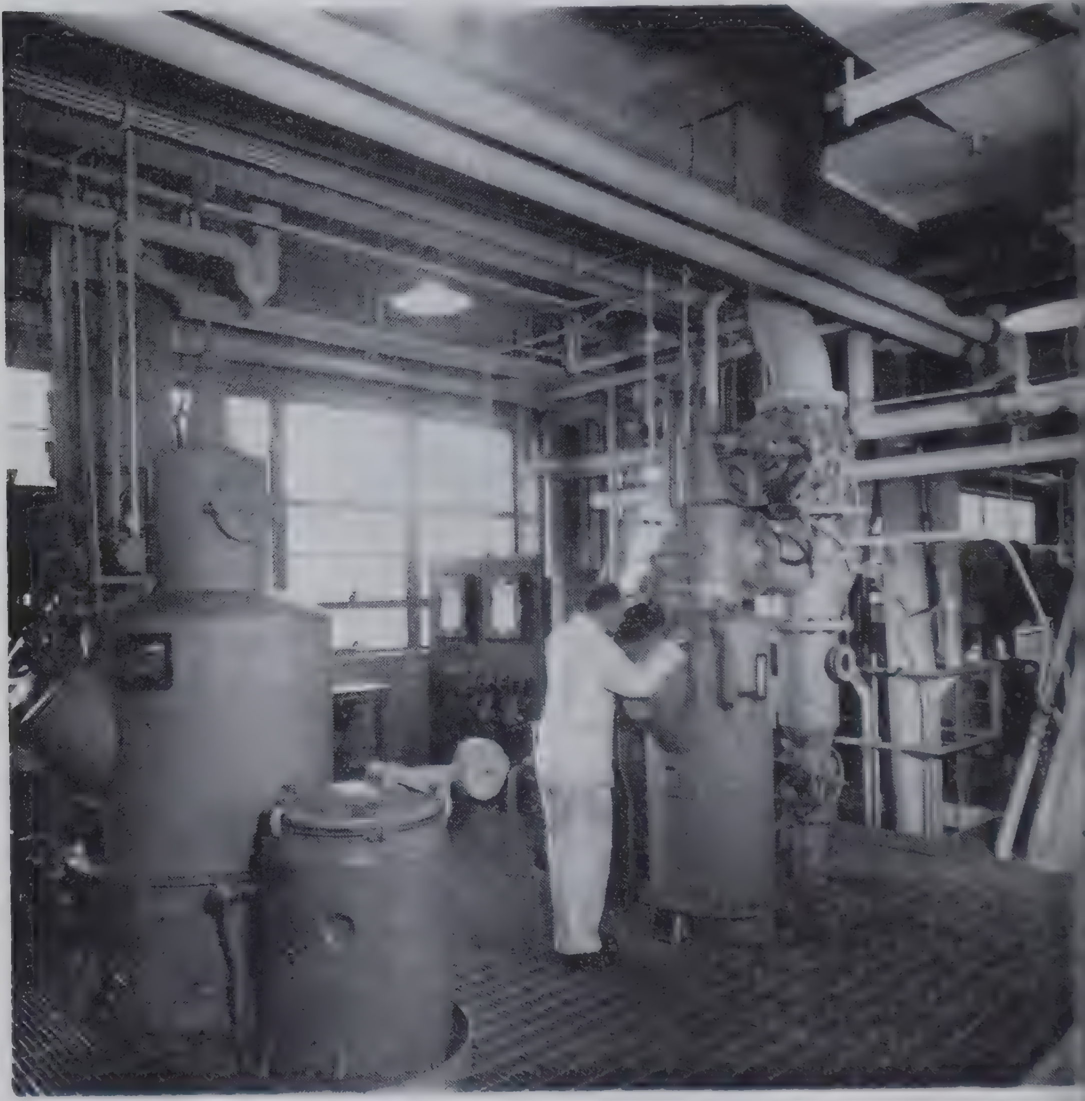


FIGURE 21b. *Vacuum Evaporators Used for the Concentration* *Crude Chloromycetin Extract* (Courtesy—Par Davis & Company)

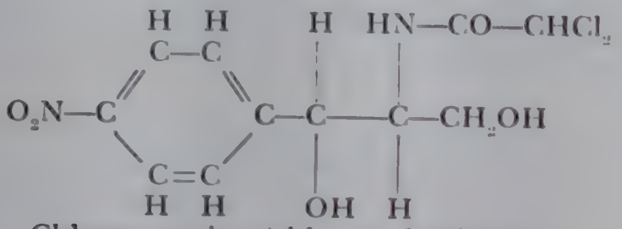
Chloromycetin has the unique distinction among antibiotic
being produced both by fermentation and by chemical synthe

present, it is believed that only 20% or less is produced by fer-ntation.[165b] It is possible that synthesis will ultimately displace fermentation process.

Some of the plant equipment used in the fermentative produc-n of Chloromycetin is shown in Figures 21a and 21b.

RMULA AND PROPERTIES[22,66,72,169,222]

The molecular formula for Chloromycetin is $C_{11}H_{12}O_5N_2Cl_2$, l the structural formula:



Chloromycetin (chloramphenicol)

The structural designation for Chloromycetin is D-(—)*threo*-lichloroacetamido-1-*p*-nitrophenyl-1,3-propanediol, which is the sis for the shorter term chloramphenicol.

Probably the most distinctive feature of its structure is the tro group; it is, as far as the reviewers know, the first organic mpound of biological origin that contains a nitro group. It is t unthinkable, of course, that organic nitro compounds exist, nporarily at least, in nitrification processes. The presence of lorine is also noteworthy, although a number of mold products ntaining nonionic chlorine have already been isolated. Since it s two asymmetric carbons, there are three other isomers, but none these has significant antibiotic activity.

Chloromycetin is a neutral compound that crystallizes as color-s needles or elongated plates and melts sharply at 150.1°C. It is tically active, $[\alpha]_D^{25} = -25.5°$ in ethyl acetate, and in water, it s a characteristic single absorption maximum at 278 mμ. It is t very soluble in water, 0.25 g per 100 ml, but is readily dissolved methanol, ethanol, butanol, acetone, ethyl acetate, and several ner organic solvents.

TIVITY[4c,64,106a,168a,180,240,304]

Chloromycetin is relatively inactive against Gram-positive bac-ia, e.g., *S. aureus,* but is very active against Gram-negative cteria, e.g., *E. coli,* and thus attacks many of the bacteria not ected by penicillin. It is very potent against bacteria producing certain intestinal diseases, e.g., typhoid and dysentery, and has give some promise in the treatment of brucellosis in humans. Its mos notable characteristic, however, is its potency against certai rickettsial diseases, e.g., epidemic typhus, scrub typhus, murin typhus, and Rocky Mountain spotted fever. It has antiviral activit against psittacosis and lymphogranuloma, but is ineffective again a number of other virus diseases. It is only moderately active again the human tubercle bacillus and is inactive against actinomycete yeasts, fungi, and the malaria parasite. Cross resistance to Aure mycin and Terramycin has been noted in some organisms resista to Chloromycetin.

TOXICITY AND METABOLISM[91,106b,119,125,168a,180,238,239,240]

Chloromycetin has a rather low toxicity. The intravenous LD for mice is about 245 mg per kg of body weight. Oral administr tion of 200 mg per kg a day to dogs over a period of 4 months ha no cumulative toxic effect on organs or tissues.

Certain blood disorders (e.g., aplastic anemia, in which t bone marrow fails to produce red blood corpuscles with possib fatal results) have been observed in connection with the prolong use of Chloromycetin. This has prompted the Food and Dr Administration to require a warning to be printed on the label follows: "Blood dyscrasias may be associated with intermittent prolonged use. It is essential that adequate blood studies be mad The nitrobenzene structure in Chloromycetin has been regarded some as the possible cause of this toxicity, but there is as yet agreement on this point. Approximately 90% of the administer dose is excreted as an inactive nitro compound and 10% as u changed Chloromycetin in the urine in 24 hours. Liver and kid tissues form inactive nitro compounds from Chloromycetin and duce the nitro group to give aryl amines. An enzyme preparation been obtained from cells of *Proteus vulgaris* and *Bacillus subt* that hydrolyzes the amide linkage, thus liberating the correspond amine and dichloroacetic acid. Both of these products were isola from the enzymic digest and identified by chemical means.

Circulin

PRODUCTION

This antibiotic is produced by an aerobic spore former, de ted as *Bacillus circulans;* this is the basis for the name cir- lin.[191,192] Production has been studied in vessels ranging from aken flasks to 2,000-gal tanks. In a dextrin-yeast-ammonium sul- e-salts medium, about 2,100 units per ml were obtained in 3 ys in flask fermentations.[192] An improved medium containing xtrin, ammonium sulfate, rolled oats, and salts gave 5,000 to 00 units (about 1 mg) per ml in 100-gal fermentors under opti- al conditions of aeration.[194] Since the pure circulin sulfate has an tivity of about 6,000 units per mg, the circulin content of the oth in 100-gal fermentations was about 1 g per l, which is a gh yield.

Circulin is obtained from the broth by adsorption on Darco 60 at pH 6.5 and elution with 25% aqueous tertiary butyl al- hol at pH 2.5 to 4.0. Chromatography on Darco G-60 with ter- ary butyl alcohol has given fractions analyzing 5,500 to 6,500 units r mg.[207] Pilot-plant runs of the antibiotic have been processed by ecipitation with a sulfonated type of wetting agent, extraction th acidified acetone, and a second precipitation at higher concen- ations of acetone. After solution in water, the product was ophilized from the frozen state and gave a powder of about % purity. The recovery from beer to powder[194] was about 44%.

HEMICAL PROPERTIES

Circulin is a basic polypeptide that has been isolated as the sul- te, hydrochloride, picrate, helianthate and reineckate. The sulfate a white, amorphous solid that melts at 226° to 228°C, is levorota- ry with a specific rotation of −61.6°, and by paper chromatogra- y behaves as a homogeneous substance. On hydrolysis, it yields leucine (10%), L-threonine (12%), L-α,γ-diaminobutyric acid (55 65%), and an optically active fatty acid, isomeric with pelargonic id (10%). From these data, circulin appears to contain one olecule each of leucine, threonine, and fatty acid and five mole- les of α,γ-diaminobutyric acid; it thus would have a minimum olecular weight of 854. It does not contain any free carboxyl oups and is apparently cyclic in character. The γ-amino groups the diaminobutyric acid are all free and impart to the peptide basic property. It is slowly inactivated by lipase (80% in 5 days); erefore, the fatty acid is believed to be attached through the hy- oxyl group of threonine. (In the polymyxins, the fatty-acid residue is assumed to be linked to one of the five γ-amino groups the diamino acid as an acyl derivative.)

Since circulin bears such a striking resemblance to the pol myxins, a comparison was made with respect to the action of lipa on circulin and on polymyxins A, B, and D. Only polymyxin B w inactivated by lipase and then more slowly than circulin. Pol myxin A, which contains the same amino acids as circulin, remain stable to lipase.[207] Because of its strong resemblance to the pol myxins, the question naturally arises as to whether circulin shou not be called a polymyxin.

ACTIVITY AND TOXICITY

Circulin, like the polymyxins, is strongly active against m Gram-negative bacteria and relatively inactive against Gram-positi microorganisms. An exception to Gram-negative activity is report by Bliss and Todd,[37] who found that circulin and polymyxins and D had little or no activity against their strains of *Prote* Circulin and polymyxin B were about equally effective in prote ing mice against infection with *Klebsiella pneumoniae* and b were much more potent than streptomycin, Aureomycin, or Chlo mycetin.[37] Circulin is absorbed from the digestive tract in dogs, not in mice.[265]

The LD_{50} dose of circulin for mice is 10 mg per kg by int venous administration and 77 mg per kg by the subcutaneous rou It is much more toxic than polymyxin D, but seems to be less jurious to the kidneys.

Erythromycin

From broths of *Streptomyces erythreus* fermentations, resea workers of Eli Lilly and Company have obtained a new antibi which acts on many Gram-positive bacteria, the typhus ricketts some large viruses, and a few Gram-negative microorganisms. thus covers the range of penicillin and part of that of the "br spectrum" antibiotics. It is being produced commercially by Lilly and Company under the trade name "Ilotycin,"[182a] by Ab Laboratories as "Erythrocin,"[10b] and by the Upjohn Compan erythromycin, which is its generic name.

It can be produced by submerged fermentation in a soyb corn steep-glucose medium maintained at 26° to 30°C. Yield out 350 μg per ml have been obtained in a typical fermentation 100 hours. Erythromycin production began at about 20 hours d continued uniformly to the end of the fermentation. The ucose concentration fell steadily during the first 60 hours and en leveled off. The pH of the medium rose from 6.4 at the start about 7.2 in 90 hours. Erythromycin is obtained from the filtered oth by extraction at pH 9.0 with amyl acetate, transferring the tibiotic into water at pH 4.0, and concentration of the aqueous lution to about 10 mg of erythromycin per ml. If the aqueous lution is adjusted to pH 9.0, the free base separates in crystalline rm. It can be recrystallized from a water-acetone (2:1) solu- on.[1,53a] A second clinically useful antibiotic, erythromycin B has een found in the broth by Pettinga, et al.[208a] A transfer of the cul- ire has been deposited with the Northern Regional Research aboratory, Peoria, Illinois, under the number NRRL 2338.[53a]

HEMICAL PROPERTIES AND COMPOSITION[10b,53a,64a,106a,128c,182a]

Erythromycin is a basic compound which readily forms salts ith acids. It has been used in the form of the crystalline base and s the hydrochloride. It is extremely soluble in alcohols, acetone, hloroform, and ethyl acetate; moderately soluble in ether; and less luble in water. It is stable in solution at pH 6 to 8, but below r above this range, it loses its activity in 24 hours.

It is levorotatory with a specific rotation of −78° and exhibits single weak broad absorption band at 280 mμ in the ultraviolet pectrum. From the elementary composition, an empirical formula $_{37}H_{65}NO_{13}$ has been calculated. The molecular weight calculated rom this formula, 732, appears probable by comparison with that btained from titration data, 725. On acid degradation, erythro- ycin yields propionaldehyde, propionic acid, an amino ugar, desosamine (3-dimethylamino-4-desoxy-5-methyl aldopentose, $_{8}H_{17}NO_{3}$), a second sugar, cladnose ($C_8H_{16}O_4$) and other unidenti- ed products.

CTIVITY[1,10b,126a,129a,148a,170c,210b]

Erythromycin has been shown to be active against Gram-positive acteria and to some extent against a few Gram-negatives. It also as potency against mycobacteria, typhus rickettsiae, and against me of the larger viruses. It is not effective against *E. coli*, *A. aero- enes*, and members of the proteus, salmonella, and shigella groups of bacteria, nor against yeasts and molds. It is active against man organisms that have become resistant to penicillin and streptomycin though such organisms may become resistant to erythromycin also The situation seems to be a race between development of resistanc by bacteria and development of new antibiotics by man. Erythromycin is administered by mouth in doses of 200 to 500 mg at 4 to hour intervals.

TOXICITY[7a,10b]

Careful testing preliminary to clinical use indicates a lo toxicity for erythromycin. The LD_{50} for mice, rats, rabbits, an hamsters was above 3,000 mg per kg in oral administration, an for mice, above 2,500 mg per kg by subcutaneous injection.

In the seventy cases treated to date of writing side effects wer rare and seemed to occur where the single doses (0.5 g) had bee exceeded. Nausea, vomiting, and diarrhea were observed in occ sional patients. Producers, however, give warning of the necessit of watching carefully for side effects of this new drug in cases whe continued or repeated courses of erythromycin are administere

Fumagillin

An unusual antibiotic, which when first discovered[127c] in 194 was notable only for its activity against the bacteriophage *Staphylococcus aureus*, but which has since then been raised to co siderable importance when it was found by McCowen et al.[179b,17] that it is a potent amoebicide. This antibiotic was called "H when first discovered, because it was obtained from *Aspergill fumigatus* H_3; but it was later given the name "fumagillin," und which designation it is being produced commercially. Fumagill is apparently identical with an antibiotic reported under the o name of phagopedin sigma. It was obtained from a mold lat identified as *Aspergillus fumigatus*.[16a]

PRODUCTION

Pilot-plant production of the antibiotic has been described Eble and Hanson.[90a] Fifteen hundred gallons of dextrin-corn ste medium were seeded with a 5% volume of a 48-hour vegetative cu ture of *A. fumigatus* H_3 that had been grown in the same mediu After fermentation for 42 hours, the beer assayed 112 μg per ml fumagillin. It was filtered with Dicalite, defatted with Skellysol

extracted with chloroform, and the extract concentrated to dry-
ss. The residual sirup was dissolved in acetone. Of the 1062 g
solids contained in this solution, approximately 210 g were
nagillin.

Crystalline fumagillin was obtained from the solution by con-
ntrating to a thick suspension at room temperature under a
trogen atmosphere, cooling the suspension under nitrogen for
hours at —30°C, centrifuging, and washing the crystalline sub-
ance five times with a total of 7,590 ml of tertiary butyl alcohol.
was then recrystallized from 3,000 ml methanol and 800 ml
ater, and 19.8 g of light-yellow crystals obtained.

HEMICAL NATURE AND PROPERTIES

Eble and Hanson[90a] have determined the elementary composi-
on (C,H,O,); melting point 189° to 194°C; molecular weight 436;
ptical rotation $[\alpha]_D^{25} = -26.6°$ (*c*, 0.25 in methanol); ultraviolet
nd infrared spectra of the compound and the chemical properties
some of its derivatives, e.g., methyl ester, bromine addition
roduct, and 2,4-dinitrophenylhydrazone. From all these data, they
ached the tentative conclusion that it is a monobasic acid with
ur conjugated double bonds, the remaining oxygen being partly
ccounted for as alkoxyl, dicarboxyl, or α-hydroxycarbonyl groups.
he empirical formula proposed is $C_{27}H_{36}O_7$. Schenck et al.[233a]
ave obtained decatetraenedioic acid, $HOOC-(CH{=}CH)_4COOH$,
om fumagillin by alkaline degradation and conclude that the
ntibiotic is a monoester of this acid, $[C_{16-17}H_{25-27}O_3]-O-CO-(CH$
$=CH)_4COOH$.

CTIVITY

Discovered as active against the bacteriophage of *S. aureus*,
was tested for antibacterial, antifungal, and antiviral potencies
nd had none of these. It sprang into considerable importance as
antibiotic when it was shown to be a potent amoebacide. Mc-
owen et al.[179a] cleared rats of *Endamoeba histolytica* in 2 days by a
tal dosage of 11 mg per kg, and Hrenoff and Nakamura[140a] cured
onkeys of amoebiasis by dosages of 50 to 125 mg per kg a day.
his activity seems to be specific for amoebae; the antibiotic had
effect on trypanosomes, spirochetes, and other parasites tested.

OXICITY

The toxicity of fumagillin is low. Eble and Hanson[90a] found the LD_{50} for mice to be 800 mg per kg in subcutaneous administra tion, while 2,000 mg per kg were tolerated orally. Hrenoff an Nakamura[140a] found no toxicity in their experiments with monkeys although bromosulfein tests indicated that a dosage of 125 mg pe kg a day might cause liver damage.

Magnamycin (Carbomycin)

Magnamycin is the trade-mark name and carbomycin th generic name of a new antibiotic, put on the market by Cha Pfizer and Co., Inc.[256a] It is produced by a streptomyces calle *Streptomyces halstedii* and can be obtained from the clarified brot (100 μg Magnamycin per ml) by extraction with methyl isobuty ketone. After concentrating the extract *in vacuo,* the antibioti is transferred into water by extracting at pH 2.0. The aqueou solution is washed with benzene, adjusted to pH 6.5, and then e tracted with ether. The ether phase is dried and evaporated to gi crystalline Magnamycin. A second crystallization from 50 methanol gave a product of 99.2% purity.

CHEMICAL PROPERTIES[88a,222a,267d]

Magnamycin is a monobasic compound, having the tentati empirical formula $C_{41-42}H_{67-69}NO_{16}$ and a molecular weight of a proximately 860. The homogeneity of the base has been establishe by solubility analysis, countercurrent distribution, and pape chromatography. It is levorotatory, $[\alpha]_D^{25} = -58.6°$ (*c*, 1% in chl roform), and melts with decomposition at 212° to 214°C. The ba is readily soluble in organic solvents, but is almost insoluble i water and hexane. It forms stable, crystalline salts of which t hydrochloride is water soluble. The ultraviolet absorption spectru shows a high, broad peak at 238mμ and a low, weak peak at 327 m

Alkaline degradation has given 1 mole each of acetic acid, is valeric acid, and dimethylamine. Mild acid hydrolysis yields t isovaleric acid ester of a sugar and an unidentified crystalline su stance, $C_{29-30}H_{47-49}NO_{12}$. The sugar, called mycarose, is an unusua branched-chain didesoxyaldohexose, $C_7H_{14}O_4$. It is apparently muc like the sugar obtained from erythromycin. In chemical compos tion, physical properties, and microbial activity, erythromycin an Magnamycin are strikingly similar. When the structural chemist of the two antibiotics has been worked out, these analogies wi obably be made clear. The situation is remindful of that which isted before the structures of Aureomycin and Terramycin were ravelled.

TIVITY AND TOXICITY[96a,96b,111a,112a,287b,299a]

Magnamycin acts mainly on Gram-positive bacteria and has tle or no activity on the Gram-negatives. Like erythromycin, it ts on rickettsia and large viruses. Microorganisms resistant to ythromycin also show some cross-resistance to Magnamycin and ce versa. Magnamycin has no cross-resistance with the older mmercial antibiotics and should prove valuable in the treatment the many cases now resistant to these antibiotics. It is administred to patients by mouth in divided doses totaling 1 to 2 g daily.

The toxicity of Magnamycin hydrochloride for mice by intranous administration is given as LD_{50}, 550 mg per kg of body eight.

Neomycin

URCE

Neomycin is an antibiotic recently discovered by Waksman and echevalier;[277] it is being manufactured by several companies. It is roduced by the discoverers' *Streptomyces* sp. 3535, a microganism closely related to *Streptomyces fradiae.* Neomycin consts of several components; therefore, it is often spoken of as the eomycin complex.[253] It is active against both Gram-positive and ram-negative bacteria.

RODUCTION

Antibiotic production in shaken flasks, amounting to 900 units er ml,[278] was obtained in a soya peptone-glucose-meat extractdium chloride-zinc sulfate medium in 144 hours. Peptone and nc were particularly effective in producing high yields. The edium was adjusted at a pH of 6.5 to 6.8 and incubated at 26° 28°C, with continuous shaking of the flasks. In a 6,000-l fermention, 2.25 g per l have been reported in 90 hours.[162a] One unit of eomycin is the minimum amount of antibiotic in 1 ml of nutrient gar that is required to inhibit the growth of *E. coli* ATCC 9637. ssuming an average of 250 units per mg for the mixed antibiotics, 0 units per ml on a weight basis is 3.6 g per l which is a large yield.

Purification and concentration of the flask product was effecte
by adsorption on and successive elution from Decalso and Dar
G-60, precipitation as the picrate, and conversion to the hydr
chloride to give a substance having a potency of 212 units per mg.[2]
Purification of the 6,000-gal batch was accomplished by acidifyin
the broth with sulfuric acid to pH 2.7, filtering off the mycelial cak
adjusting the filtrate to pH 8.0, and adsorbing on Darco G-60. Th
neomycin was eluted from the carbon with 10% acetone at pH 2
and precipitated with more acetone. The crude neomycin sulfa
was further purified by chromatography through a carbon colum
The neomycin sulfate thus obtained appeared homogeneous b
paper chromatography and solvent-distribution tests. It analyze
1,000 μg per mg by the streptomycin *B. subtilis* assay.

Further purification of neomycin has been effected by precip
tation with picric acid, chromatography through alumina, an
crystallization as salts of different sulfonic acids, e.g., *p*-(*p*′-hydrox
phenylazo)-benzene sulfonic acid. A crystalline sulfonate, desi
nated neomycin A, has been obtained from crude neomycin
Peck et al.[204] Recrystallization of the sulfonate from aqueo
methanol gave a product with constant properties. The hydr
chloride was prepared from the sulfonate by precipitation fro
butanol-hydrochloric acid with acetone. It had an activity of 1,7
units per mg by a cup diffusion assay with *B. subtilis* and 50 un
per mg by *E. coli* turbidimetric assay.[300]

A second component, believed to make up much of the activ
of the broth and designated neomycin B, has been obtained as t
crystalline helianthate and the *p*-(*p*-hydroxyphenylazo)-benze
sulfonate by Regna and Murphy.[223] Sulfates prepared from t
crystalline dye salts behaved as homogeneous substances when su
jected to paper chromatography and had an activity of 220 un
per mg by turbidimetric assay with *B. subtilis* and 255 units
K. pneumoniae turbidimetric assay.

Two homogenous antibiotics, neomycins B and C, have be
obtained by Dutcher et al.[87] The B compound appears to be
same as the neomycin B of Regna and Murphy. Neomycins B an
were isolated by chromatography of the hydrochlorides in 8
methanol over alumina. Crystalline reineckates, picrolonates a
p-(*p*-hydroxyphenylazo)-benzene sulfonates have been obtained
both components.

The potencies (units per milligram) of neomycins A, B, an

r greatly, depending on the method of assay, even with the
e test organism. By cup diffusion assay, A is two to three times
otent as B or C, but by turbidimetric assay, it is only about
tenth as active.[78,127b] This shows that A diffuses readily through
r whereas B and C do not. The difference in diffusion is prob-
y related to the smaller size of the A molecule. For comparison
otency, the turbidimetric method gives a truer picture as then
re is no problem of the antibiotics coming in contact with the
organism.

Swart, Lechevalier, and Waksman[253a] have examined five prep-
tions of neomycin, one of their own and four made by antibiotics
ducers. These samples were tested in a Craig countercurrent
aratus and only one preparation, neomycin A, appeared to be
ly homogeneous. The others seemed to consist of three to four
in components and several small fractions.

Contradictory results obtained by different investigators prob-
y arise from the lack of a homogeneous standard, different
thods of assay, and different systems of purification. The use
different methods is really desirable, as what appears homoge-
ous by one method may show up as a mixture by another pro-
ure. The confusion that now exists will probably be cleared up
better methods of fractionation are developed and the chemistry
the different components is ascertained.

A quite different antibiotic, fradicin, is also produced in the
omycin fermentation.[254] The distinguishing feature of this anti-
tic is its activity against fungi. Up to 69% of the antibiotic
ivity of the broth may consist of the antifungal agent if fermenta-
n conditions are regulated to favor its production. Low aeration,
reased amounts of soya peptone, and addition of glucose to the
dium increased the production of fradicin. It is obtained from
broth by extraction with butanol, purified through ethanol and
tone, separated as oil from petroleum ether and then lyophilized
give a tan-colored powder. Fradicin has been obtained in crystal-
e form by Hickey and Hidy[132] who, on the basis of elementary-
nposition and molecular-weight determinations, proposed the
pirical formula $C_{20}H_{33}N_{3}O_{4}$. Fradicin is a weak base, which has
ecific rotation of + 65° and an ultraviolet-absorption spectrum
h a prominent peak at 293 mμ and several other smaller ones.
ibition of several pathogenic fungi by the crystalline compound
uired 1 to 10 μg per ml. Acute toxicity tests by intraperitoneal

injection of mice gave an LD$_{50}$ value of 4 mg per kg of body w

CHEMICAL NATURE AND PROPERTIES

Although the elementary composition of neomycin has determined a number of times, no empirical formula has bee ously considered for any of the components. The reluctance of tigators to offer empirical formulas is perhaps based on doub the homogeneity of the different preparations. Dutche Donin[illegible] suggested that neomycins B and C appeared to hav empirical formula $C_{23}H_{46}N_6O_{13}$, but the evidence for this fo does not seem to be complete or convincing.

All the neomycins are basic compounds and form sal various acids, e.g., dye sulfonate, reineckate, hydrochlorid sulfate. The neomycins contain nitrogen, all of which is p as amino nitrogen, since it is all removed by the action of n acid. The ninhydrin test is positive and the Molisch and car tests for carbohydrate residues are positive with neomycins B but negative with neomycin A. Fehling's and Tollen's te reducing substances are negative with the original neomycins C, but after hydrolysis, the tests are positive. All of the comp are dextrorotatory, but the specific rotation varies, being +83° +80° for B and +54° to +58° for C. The neomycins are ex stable to alkali, e.g., refluxing with excess barium hydroxi 18 hours did not lessen the activity of a neomycin prepa Neomycin A is very stable to acid, but B and C lose much o activity on heating with acid.

Neomycin has been degraded with acid by Leach and Te to yield a crystalline base, designated neamine. The base s some antibiotic potency, about 6% of that of the original neo In a later paper, Leach and Teeter[illegible] compared neamin neomycin A and found the two to be identical. Only a mont Dutcher and Donin[illegible] reported that a methanolysis produc they had previously obtained[illegible] from neomycins B and C w same as neomycin A.

By drastic hydrolysis of neomycin A, Kuehl et al.[illegible] obta degradation product, $C_6H_{14}N_2O_3$, which they determined 1,3-diamino-4,5,6-trihydroxycyclohexane and established its st unequivocally by synthesis. Leach and Teeter obtained th cyclohexane from neamine. The question naturally arise

r component, if any, is contained in neomycin A, neamine,
methanolysis product.
her et al.[37] reported two other hydrolysis products from
s B and C. One of these appeared to be a diaminodesoxy
d the other had the properties of a pentose. The presence
components in neomycin suggests a pattern similar to that
streptomycin. The cyclohexane base of neomycin is closely
the streptidine of streptomycin, the pentose of neomycin
espond to the streptose of streptomycin and the diamino-
cose of the new antibiotic may parallel the N-methyl-
ine of the older one. Bacterial spectra and development of
resistance lend some support to the chemical data pointing
ionship between the two antibiotics, but final decision on
nt of this relationship must wait until the structure of
ycins has been determined.

mycin is active against numerous Gram-positive and Gram-
bacteria, including the tubercle organism.[136a,267a,270,275,276,277]
do not develop resistance to neomycin as readily as to
ycin and strains that are resistant to streptomycin are de-
by neomycin. Resistance to neomycin can be developed
d such strains seem to have some resistance to streptomycin.
reverse effect does not seem to occur. On a weight basis,
n and streptomycin have about the same activity.[267]
vo tests (mice, chick embryo, guinea pigs) have shown it
fective in combatting infections with *S. aureus*, *S. schott-*
S. pullorum, *E. typhosa*, *K. pneumoniae*, *P. vulgaris*, and
rculosis.[137,152,217,266] In humans, many cases of urinary and
fections, refractory to other antibiotics, have been treated
lly with neomycin.[83,162,136e,136a] In the treatment of tubercu-
omycin seems to be inferior to streptomycin. However, it
seful in cases that are resistant to streptomycin.[136e,266]

y

mycin is a toxic substance, but there still seems to be some
as to how great is its toxicity. Loss of weight and kidney
n guinea pigs treated with neomycin for tuberculosis have
orted.[152,246] Kidney damage[267a] and progressive deafness[36a]
ns after neomycin therapy have been observed. Prepara-

tions containing about 200 units per mg had an LD_{50} of about
mg per kg when injected intravenously into mice.[157] By the su
cutaneous route, the hydrochlorides of pure neomycins B and
had a mouse toxicity of LD_{50} amounting to 220 mg per kg and 2
mg per kg, respectively.[127a]

Polymyxins

SOURCE

Polymyxin is a generic name adopted to indicate antibio
obtained from strains of *Bacillus polymyxa*. To date, there are
least five such antibiotics, closely related in respect to produc
organisms, activity, and chemical composition. These are nam
polymyxin A, B, C, D, and E. Originally, polymyxin A was kno
as aerosporin because it was produced by a bacterium identified
Bacillus aerosporus. This organism was later shown to be a str
of *B. polymyxa* and, at a conference of workers in this field,
more appropriate and taxonomically derived term, polymyxin,
agreed upon.[50]

Three groups of workers reported independently the existe
of antibiotics produced by *B. polymyxa:* Ainsworth, Brown,
Brownlee[8] of the Wellcome Physiological Research Laborato
England; Stansly, Shepherd, and White[243] of American Cyanam
and Benedict and Langlykke[77] of the Northern Regional Resea
Laboratory in the United States.

PRODUCTION

Of the polymyxins, B and D have been produced in shal
stationary cultures, shaken flasks, and aerated pilot-plant ta
The following brief statement applies to the production of p
myxin D in an aerated medium of 15 l volume.[244] The med
contained about 1.0% glucose, 2% $(NH_4)_2SO_4$, 0.5% yeast ext
(Difco), 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.005% NaCl
0.001% $FeSO_4 \cdot 7H_2O$ and was adjusted to pH 7.9. In previous
fermentations, 1.0% soybean in place of yeast extract gave eq
good yields. The medium was aerated at the rate of 64 l per
for 5 days at 25°C. The pH fell to 6.8 on the third day and
rose to 6.8. In fourteen runs, the polymyxin ranged from 16

verage 216) units per ml. Assuming 1,800 units per mg, the ge concentration of polymyxin is only about 0.12 g per l which her low as commercial antibiotics go.

or isolation of polymyxin D, the beer was filtered through Super-Cel, adsorbed on Darco G-60, and eluted with methaydrochloric acid. The polymyxin hydrochloride was precipi- with acetone, washed, and dried in a vacuum. The product ved 1,100 to 1,400 units per mg and the over-all recovery was 60%. The crude polymyxin hydrochloride was further puriby extraction with butanol at pH 9.0 and converted back e hydrochloride (potency 1,700 to 1,800 units per mg). A uct of somewhat higher potency was obtained by partition natography.[237]

A very potent crystalline derivative of polymyxin B has been ned by adsorption on cotton succinate, elution, precipitation Polar Yellow solution, precipitation of the free base with ylamine, and conversion to crystalline polymyxin B naphtha-3-sulfonic acid. This compound was levorotatory, $[\alpha]_D$ 53.3°, and paper chromatography showed only a single onent.[225]

The cultures producing polymyxins A and B also form small tities of other as yet unidentified polymyxins. If a number of ns of the polymyxa and related types were thoroughly studied, robable that many more antibiotic polypeptides similar to the myxins would be found.

ICAL NATURE AND PROPERTIES[23,61,148,298]

The polymyxins are basic polypeptides combined with a fatty ($C_8H_{17}COOH$). They all contain L-threonine, the C_9 fatty nd a new amino acid, L-α,γ-diaminobutyric acid. (Some of form of this acid was obtained, but this may have been pro- by racemization during hydrolysis.) They differ in respect other amino acids found in the polypeptide. Polymyxin A es D-leucine, polymyxin B yields D-leucine and phenylalanine. yxin C gives only phenylalanine, and polymyxin D contains ne and D-serine, but no phenylalanine. The composition of yxin D is 5 moles of L-α,γ-diaminobutyric acid, 3 moles of ine, 1 mole of D-leucine, 1 mole of D-serine and 1 mole of fatty which gives a molecular weight of about 1,150. The amino acids appear to be arranged in the form of a closed cycle. fatty acid apparently is linked to one of the diaminobu acid residues through the γ-amino group in the side cha form an acyl derivative. The γ-amino groups of the other diaminobutyric residues are free and give basicity to the pound. The fatty acid has not been fully characterized by synt but degradation studies indicate it is (+)-6-methyloctanoic, $CH_3CH_2CH(CH_3)CH_2CH_2CH_2CH_2COOH$. The acid appears identical in all the polypeptides, although the optical activ the acid from polymyxin A,[84] $[\alpha]_D = +8.2°$, seems to differ that obtained from polymyxin B,[100] $[\alpha]_D = +32°$.

The various polymyxins differ in other respects, e.g., bility in butanol, titration curves, absorption spectra, and ch tographic behavior. Their antibiotic properties are unaffec digestion with pepsin, trypsin, or papain.

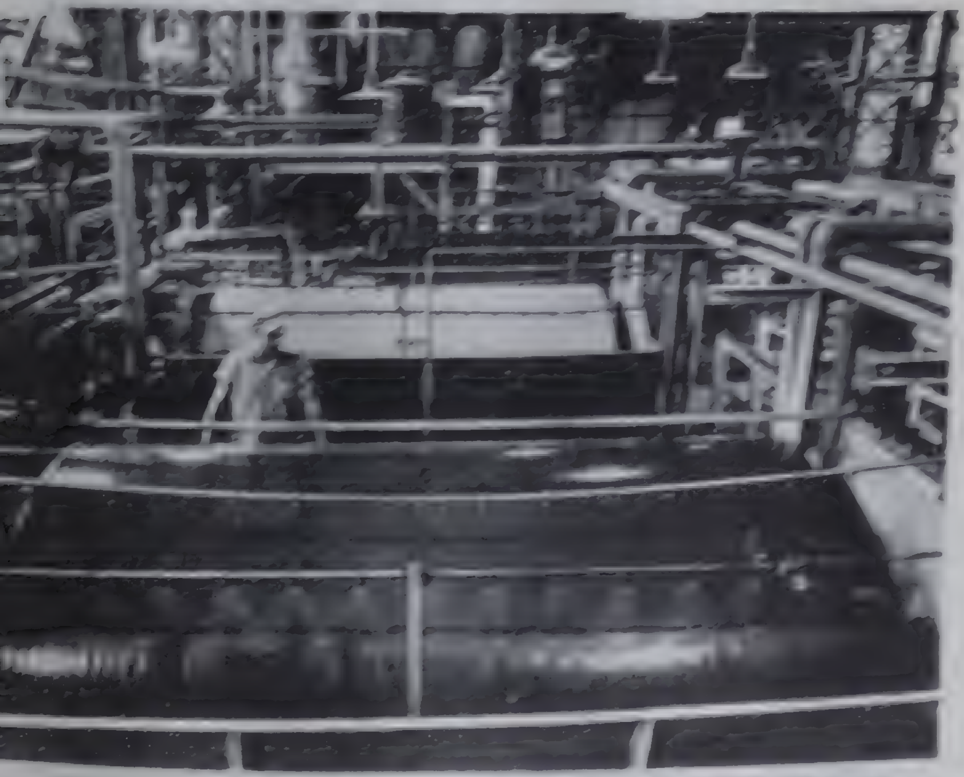
ACTIVITY AND TOXICITY[illegible]

The polymyxins are distinctive in being strongly active a Gram-negative bacteria and largely inactive against Gram-p organisms. They have been tested clinically against typhoid, g enteritis, urinary infections, and whooping cough. Polymyxin peared unusually promising against the organism, *Hemo pertussis*, associated with whooping cough, but unfortunately proteinuria seems to accompany its use and the antibiotic a less promising than formerly. All of the polymyxins appear to renal damage of varying severity and duration, but this is leas polymyxin B. Administration of methionine, or choline, taneously with polymyxin A, prevented proteinuria in rats an Methylating or acetylating the antibiotic accomplished the result. However, the author did not test the modified prod see if it still had antibiotic activity. If activity is retain toxicity is removed, modification should greatly extend the ness of the polymyxins. In spite of the danger of causing d they have proved very useful in the treatment of refractory tions, such as *Proteus* and *Pseudomonas*, which often rem affected by other therapeutic agents.

The mean LD_{50} for polymyxin A is 6.9 mg per kg intrav and 13.9 mg per kg intraperitoneally. Polymyxin B has the same toxicity as the A form, but D is only one half to one as toxic as A and B.

Terramycin

mycin is an important commercial antibiotic manufac-
Chas. Pfizer and Co., Inc. It is produced by a new actino-
treptomyces rimosus, so-named because of the cracked
e of the colonies on agar plates.[194,241] A culture of the or-
s been deposited with the Fermentation Division of the
Regional Research Laboratory, Peoria, Illinois, under the
RRL 2234. Terramycin was discovered in the course of
ve screening program that in many years of testing cov-
hundred thousand soil samples from all parts of the
It is evident that much effort and expense is needed to
w and useful antibiotic. Of course, during this search,
many other new, but not useful, antibiotics were found by
erers of Terramycin. Although there are many papers
ith the antimicrobial action and clinical evaluation of the
only a short paper[194] and the patent[263] give information
duction and isolation.



. *Filtration of Terramycin Fermentation Broth* (Courtesy—Chas. Pfizer & Co., Inc.)

PRODUCTION

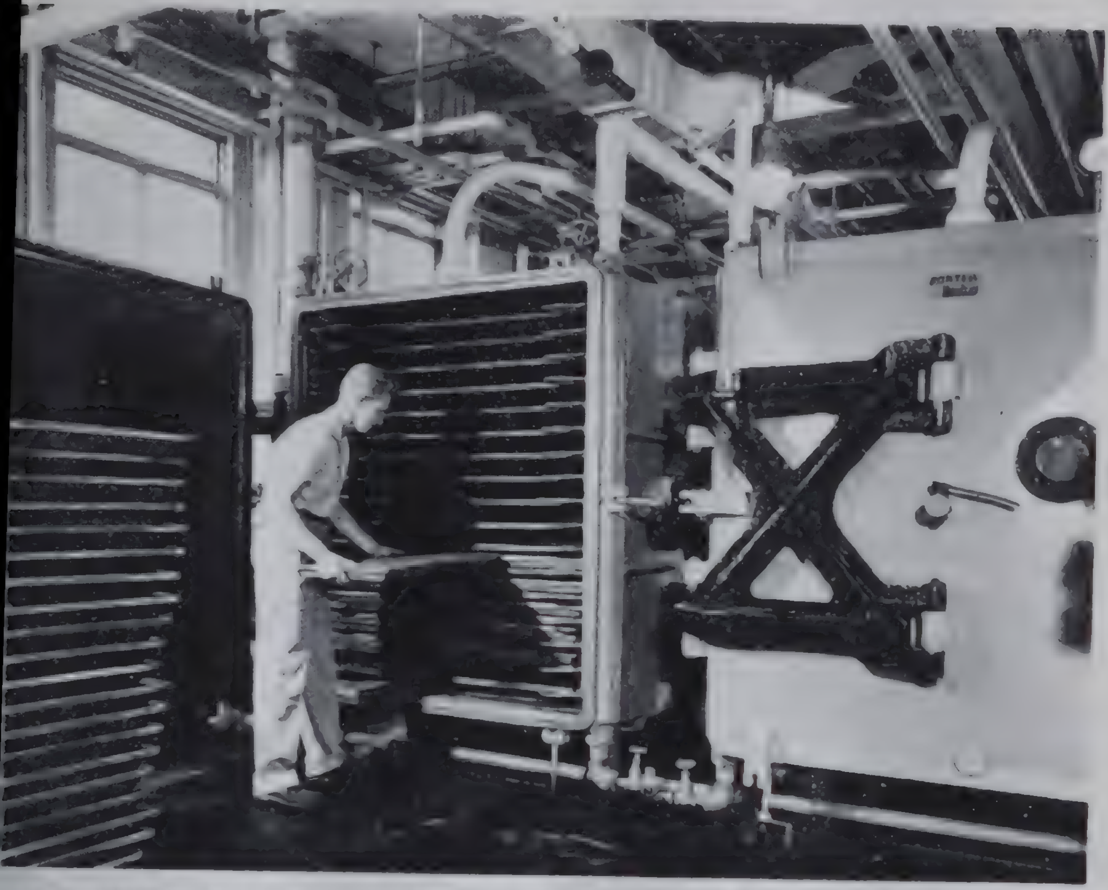
A number of different media are given in the patent[241] as being suitable for production of Terramycin. A typical medium contains 3% soybean meal, 0.5% corn starch, 0.1% N-Z-Amine B (an enzymic digest of casein), 0.3% sodium nitrate and 0.5% calcium carbonate. It is adjusted to pH 7.0 and, after sterilization, is seeded with a 2.5% by volume inoculum of the organism. Incubation with aeration and agitation is carried out at 24° to 30°C for about 48 hours and then the broth is processed. Yields given in the patent are expressed as coli dilution units (CDU) and range from 280 CDU per ml in 400-gal tanks to 2,560 in 1-gal glass fermentors. In shaken flasks, 640 CDU per ml are reported. A coli dilution unit is the quantity of Terramycin in 1 ml that is required to inhibit the growth of *E. coli* for 18 hours under specified conditions of inoculum, medium, and temperature. It is not possible to express these figures on a weight basis, as the quantity of pure Terramycin in 1 CDU is not given in the patent. Judging from another paper[13] on the inhibition of *E. coli* by crystalline Terramycin in liquid medium, the yields must be well over 1 g per l. No information ha been published as to chemical changes in the medium during fermentation except that the pH rises from 7 to 8.

Terramycin can be isolated from the broth by a number o methods. One of those given in the patent involves filtering off th mycelium, adsorbing the Terramycin at pH 7.0 on Norite, elutin with water saturated with butanol at pH 1.5, transferring first t butanol at pH 9 and then back to acidified water. On neutralizin to pH 7.5, a precipitate of crude Terramycin is formed. Crystallin Terramycin can be obtained by further purification and final crysta lization from water or methanol as the free base, the hydrochlorid or the sodium salt. An effective means of purification is a Cra counter-current distribution with butanol and water at pH 3. Th crystals of the free base are white and those of the hydrochlori and sodium salt are lemon yellow.

Equipment used in the filtration of Terramycin broth is show in Figure 21c.

Crystalline Terramycin is dried in ovens such as that show in Figure 21d.

A second antibiotic, rimocidin, is produced by *S. rimosus.*[74b,2



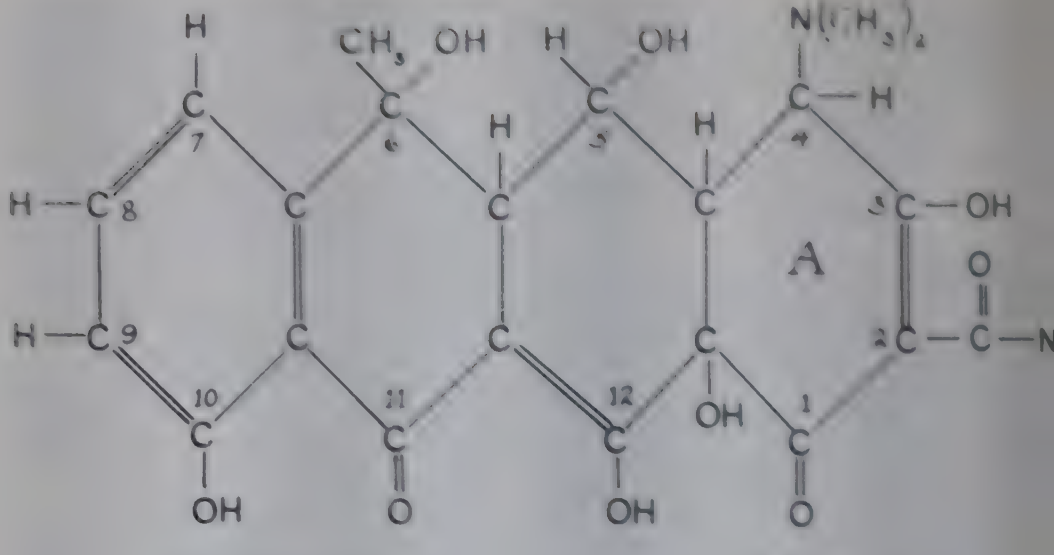
GURE 21d. *Loading a Drying Oven with Crystalline Terramycin*
(Courtesy–Chas. Pfizer & Co., Inc.)

is retained in the mycelium, instead of being secreted into the edium, and is obtained by extraction of the mycelium with tanol. The crystalline sulfate has been prepared and has the lowing percentage composition: C, 57.65; H, 7.82; N, 1.81; S, 03. It is dextrorotatory, $[\alpha]_D = +75.2°$, melts at 151°C with composition, and shows ultraviolet absorption at 279, 291, 304, d 318 mμ. The infrared absorption band shows many maxima.

The most distinctive antibiotic feature of rimocidin is its anti- ngal activity and lack of antibacterial potency. Inhibition of a mber of pathogenic fungi has been demonstrated at concentra- ns of 1 to 5 mg per ml. It has some, but not striking, activity inst protozoa, e.g., *Endamoeba histolytica.* The mouse toxicity rimocidin (LD$_{50}$) is 20 to 30 mg per kg of body weight injected ravenously.

EMICAL NATURE AND PROPERTIES[104,224,241,247]

As has already been pointed out (p. 302), Terramycin has the ne structural formula as Aureomycin except that it contains no



Terramycin

$C_{22}H_{24}N_2O_9$
M.W. 460.2

chlorine and has an additional hydroxyl group at 5. The findi an antibiotic so closely related to Aureomycin and still not cov by the Aureomycin patent has been a most profitable discove one company and a corresponding loss to another. If one re on the many examples of closely related compounds that are duced by similar species or even by strains of the same spec it would not be surprising if other naphthacene antibiotics, fo ample, with a different number or distribution of chlorin hydroxyl groups were discovered among the metabolic produ other streptomycetes.

Terramycin is an amphoteric compound and crystalli the dihydrate, the hydrochloride, and the sodium and pota salts. From water, the hydrochloride comes out as platelets from methanol as needles. It has a high specific rotation, — in 0.1 N HCl. The free base melts at about 185°C, with dec sition. It is soluble in methanol, ethanol, acetone, and prop glycol, and insoluble in ether and petroleum ether. In Terramycin is readily soluble at pH 1.2 (31.4 mg per ml) and 9.0 (38.6 mg per ml). It is a very stable antibiotic. In water 2.5 and 25°C, no detectable loss occurred in 30 days. At p and 37°C, 50% of the activity remained after 26 hours and 10 and 37°C the half life was 14 hours. In the dry stat crystalline product can be stored at room temperature fo periods.

ITY[69a,135,145a,156a,166a,172,206,231a,261,305a]

'erramycin is active against a wide variety of microorganisms lisease-producing agents. It acts on most Gram-positive and negative bacteria, on rickettsiae, certain viruses, some spiros, and certain protozoa. It does not appear to be effective, or y slightly so, against the tubercle bacillus, bacteria of the pround pseudomonas types, yeasts, and fungi.

'erramycin is widely used in the treatment of pulmonary inns, including viral pneumonia, bacterial infections of the die and urinary tracts, typhus and other rickettsial fevers, the hete diseases syphilis and yaws, and the virus diseases lympholoma venereum and granuloma inguinale. Promising results been obtained in the treatment of brucellosis and malaria.

ince it is ineffective against proteus, pseudomonas, and monilia, microorganisms may develop when their natural competitors, iram-negative bacteria, have been eliminated and thus bring what is called a superinfection, that is, an infection superbed on another. Such infections are likely to occur in the inal and urinary tracts. Microorganisms that are resistant to amycin are also resistant to Aureomycin and vice versa. efore, the use of either antibiotic in cases where the other is ctive seems to be precluded.

Combinations of antibiotics are often used, but the combinamay be less effective than adequate doses of the individual iotics alone. Useful combinations among penicillin, strepto1, bacitracin, and neomycin have been noted. Members of roup may give bad results with Aureomycin, Chloromycetin, erramycin. The effect of combinations cannot be predicted. ort [156] on the subject points out the many problems in making inations and says "only if the organism proves resistant to drugs by laboratory test or by adequate therapeutic trial d drug combinations be employed."

ITY[156,172,261]

'he toxicity of Terramycin is low, the LD_{50} for mice being 200 mg per kg by intravenous injection. In clinical trials, is no indication of injury to the kidney, liver, or bone marrow.

Tetracycline*

SOURCE[37b,65c,185a]

As mentioned previously (p. 302), Aureomycin (chlorotetracycline) and Terramycin (oxytetracycline) can be regarded as derivatives of a basic structure, tetracycline. The conversion of chlorotetracycline to tetracycline can be accomplished by simultaneous dechlorination and hydrogenation. Chlorotetracycline is dissolved in a suitable solvent (e.g., methyl Cellosolve), containing a palladium-charcoal catalyst and triethylamine, and hydrogen is passed in at atmospheric pressure and room temperature. The uptake of hydrogen is rapid and in 15 to 20 minutes is virtually complete. The reaction consumes about 1 mol of hydrogen per mol o chlorotetracycline. The triethylamine neutralizes the hydrochlori acid that is produced. After removal of the catalyst, the reactio mixture is poured into water and the free base of tetracyclin crystallizes.

A later report[185a] by another group of workers contains th interesting information that tetracycline has been obtained b fermentation. The *Streptomyces* producing it was found in a sam ple of Texas soil and was isolated in the course of a search fo organisms producing new antibiotics. To date the identity o the *Streptomyces* has not been published. The authors noted tha the culture produced an antibiotic that resembled chlorotetra cycline and oxytetracycline, but differed from these in certain re spects and was evidently a different substance. High-yieldin mutants of the organism were developed and improved means o purification were devised. To date no information has been pu lished as to the medium, sequence of chemical changes during th fermentation, and yields of antibiotic. Tetracycline is also r ported[37a] to be produced in small quantities by strains of *Strept myces aureofaciens* simultaneously with chlorotetracycline. Probabl the tetracycline culture produces also some chlorotetracycline, a cultures usually produce more than one antibiotic. Details regar ing the recovery of tetracycline from the broth are not available bu because its properties are similar to those of chlorotetracycline an etracycline. probably the same general procedures could be .

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* The trade names for tetracycline are: Achromycin (Lederle Laboratories Div sion of American Cyanamid Co.), Tetracyn (J. B. Roerig, Division of Ch Pfizer and Co.), and Polycycline (Bristol Laboratories, Inc.).

MICAL NATURE AND PROPERTIES[37b,65c,185a]

Since tetracycline has the same basic structure as chlorotetra- ne, but contains a hydrogen instead of chlorine, the molecular ula is $C_{22}H_{24}N_2O_8$ and the molecular weight is 444.2. As would xpected, some of its properties are similar and others different n those of chlorotetracycline. The specific rotation is −258° .1 N HCl as compared to −240° for chlorotetracycline and 6° for oxytetracycline. The ultraviolet absorption spectrum rs markedly from that of chlorotetracycline, but is almost iden- l with that of oxytetracycline; strong peaks are present at 267 357 mμ. The infrared spectra of the three antibiotics likewise w similarities and differences. The three antibiotics can also distinguished by countercurrent distribution and color tests. h *p*-dimethylaminobenzaldehyde in dilute aqueous solution, acycline gives an orange-yellow color, chlorotetracycline canary ow, and oxytetracycline bluish green.

Another means of distinguishing the tetracyclines is by paper matography. Chlorotetracycline can be separated from the r two in a *n*-butanol-water system; and in *n*-amyl acetate satu- d with water, tetracycline can be separated from oxytetracycline.

Tetracycline appears more stable than the chloro and hydroxy vatives.

IVITY AND TOXICITY[37a,72a,96c,156e,179a,185a,214a,299b,300b]

Tetracycline has a bacterial spectrum similar to that of chloro- acycline and oxytetracycline and has much the same potency xperimental infections. Microorganisms made resistant to one acycline appear also resistant to the other two. Preliminary ical trials in human patients show a higher tolerance level for acycline and an efficiency in counteracting infections apparently l to that of the other tetracyclines.

The acute toxicity of tetracycline is about the same as that of ther tetracyclines. The LD_{50} for mice by intravenous injection 0 mg per kg.

Tyrothricin (Gramicidin and Tyrocidine)

The literature on these antibiotics is so voluminous that only a few of the many papers can be referred to in this report. For more details, the reader should consult an extensive review by Hotchkiss[139] or a brief one by Neuberger.[196]

PRODUCTION

Tyrothricin is the oldest commercial antibiotic. It is produced by several firms, but, because of its limited use, does not bulk as large in trade as a number of other, more recent additions.

Gramicidin (sometimes called D after its discoverer, Dubos) and tyrocidine are separate antibiotics that have been obtained from tyrothricin by fractionation procedures. Both have been obtained in crystalline form and, for a long time, each has been regarded as homogeneous. However, recent work[23a,123] indicates that there are several components in both gramicidin and tyrocidine. A third crystalline product, gramicidin S, has been obtained, but it comes from a different organism from that producing gramicidin D and tyrocidine.

Bacillus brevis ATCC 8185 is the organism from which tyrothricin was obtained by Dubos and associates.[80,140] Another strain o *B. brevis* was used by Gause and Brazhnikova[116] for the productio of gramicidin S. Many strains of *B. brevis* have been shown to produce antibiotics having some properties similar to those of gramicidin and tyrocidine; but they have not been sufficiently purified t indicate whether or not they are identical with gramicidin an tyrocidine. Probably they are all polypeptide mixtures and th separation and identification of the components of such mixture is an exceedingly difficult and laborious undertaking.

Tyrothricin can be produced in shallow static media and als in submerged aerated cultures. Good yields of 0.5 to 0.9 g per have been reported.[12a,139] Both synthetic and natural media hav been used. The antibiotic can be readily obtained from the mediu by precipitation at pH 4.8, extraction of the precipitate with alc hol, a second precipitation with salt solution, and fractionation o the precipitate (tyrothricin) with acetone-ether to give a solubl portion (gramicidin) and an insoluble portion (tyrocidine). Th first portion can be crystallized from acetone to give gramicidin and the second from methanol-hydrochloric acid to give tyrocidin hydrochloride. Gramicidin makes up 10 to 20% and tyrocidi about 40 to 60% of the tyrothricin product.[139]

IEMICAL NATURE AND PROPERTIES[23a,23b,120,123,128a,132b,139,255,256]

The gramicidin complex (D) acts as a neutral polypeptide, pparently cyclic in nature and, on hydrolysis, gives five amino ids and ethanolamine. It has been calculated that there are three alanine, two glycine, six D-leucine, six L-tryptophan, five DL-valine, nd one ethanolamine residues in the complex. The peptides, alanyl-D-leucine, L-alanyl-D-valine, D-leucyl-glycine, D-valyl-L-valine nd L-valyl-D-valine have been isolated from partial hydrolysates. he data are not entirely consistent, but the discrepancies may e due to the fact that the gramicidin used, although carefully ystallized, was probably not homogeneous. Gramicidin D has een calculated to have a molecular weight of about 2,800 and a elting point of 228° to 230°C. When gramicidin D was fraction- ted by the Craig countercurrent-distribution method, the major omponent, A, melted at 227° to 228°C and a second, smaller component B melted at 258° to 259°C. Gramicidin A was found to ontain somewhat more tryptophan than B. Evidence for the presence of at least two other components was obtained, but these did ot crystallize sharply and appeared to be mixtures.

Crystalline tyrocidine is a mixture of polypeptides containing t least three components, A, B, and C. By extensive fractionation n the Craig countercurrent-distribution apparatus, the main component, A, was obtained in an apparently pure state and its mino acid content and molecular weight were determined. The mino acid residues found were: two of D-phenylalanine, and one ach of L-phenylalanine, L-valine, L-tyrosine, L-leucine, L-proline, ornithine, L-glutamine, and L-asparagine. There are no free -amino or α-carboxyl groups, which indicates that the peptide as a cyclic structure. The γ- and β-carboxyls of glutamic and as- rtic acid, respectively, are present as the corresponding amides. he δ-amino group of ornithine is free and gives the peptide its asic property. The molecular weight 1,270, calculated from the nino acid content, agrees well with that (1,300) obtained by an dependent method. Tyrocidine A hydrochloride has a melting oint of 240° to 242°C and a specific levorotation of −111°.

The chemical character of gramicidin S is now thoroughly nderstood due to the work of Synge. The crystalline hydro- loride has been obtained from the cells by precipitation with id and extraction with ethanol and the free peptide has also been crystallized. It is a cyclopentapeptide (or possibly a decapeptide) with one residue each of the amino acids named, in the following sequence: -L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl-. Open-chain pentapeptides of the same structure as gramicidin S have been synthesized, but the antibiotic activity of these peptides was only about one hundredth of that of the natural compound. From this, it appears that the antibiotic potency of gramicidin S is associated with its cyclic structure. Later evidence shows that gramicidin S is a decapeptide having two basic groups (ornithine) and a molecular weight of 1,142. The decapeptide structure helps explain why the synthetic pentapeptide has so little potency.

ACTIVITY AND TOXICITY[110,115,131,206,220,231,283]

Apparently, gramicidin D is active almost exclusively against Gram-positive organisms, but it is not inactivated by serum, etc., and, therefore, it is active *in vivo* as well as *in vitro*. Tyrocidine has stronger action against a wider bacterial spectrum, but is inhibited by serum, etc., and is, therefore, inactive *in vivo*. Neither antibiotic has proved a success in subcutaneous, intramuscular, or oral administration.

Tyrocidine is the most toxic because of its high hemolytic activity. Gramicidin D is not rapidly and strongly hemolytic like tyrocidine, but it brings about a slow and delayed hemolysis that continues for many hours. Combination of gramicidin D with formaldehyde decreases its hemolytic activity by 80% while reducing its antibacterial activity about 50%.

Much less information is available on the activity and toxicity of gramicidin S. It is reported to be active against a number of Gram-positive bacteria and, like tyrocidine, is ineffective in the presence of serum. When administered intraperitoneally, 15 to 20 mg per kg of body weight was toxic to rats.

Tyrothricin is in commercial production for therapeutic use but its producers warn against any parenteral administration of the drug because it is a systemic poison when introduced into the blood stream (LD_{50} is about 3.75 mg per kg for mice intravenously) Oral administration seems to be ineffective. It is designed fo topical use only, at the site of the infection, where it can be brough into direct contact with the infecting organism. Superficial ulcers abscesses, wounds, etc., and infections in body cavities not directl connected with the blood stream usually respond to this antibioti ien the invading organisms are Gram-positive. Presence also of ram-negative bacteria may interfere with the treatment. Doses fficient for bactericidal action are not toxic to body tissues, though ere is some toxicity at higher levels. Tyrothricin has had some e in veterinary medicine, also.

Viomycin

After 3 years of testing, viomycin was approved as a new drug the Food and Drug Administration early in 1953 and has been aced on the market by Parke, Davis and Co. and Chas. Pfizer and o., Inc. Streptomyces producing this antibiotic were discovered dependently in the laboratories of these two firms and named *reptomyces floridae* and *Streptomyces puniceus* respectively. The st name indicates the source of the soil sample from which the lture was isolated and the second denotes the purplish-red color the vegetative mycelium produced by the culture.[23,103]

RODUCTION[23,65b]

Factors affecting viomycin production in shaken-flask fermentaons have been reported for *S. floridae.* The organism can use a umber of carbohydrates, but commercial glucose gave the highest tre. Several commercial nitrogenous substances gave good yields viomycin. A typical high-yielding medium consisted of 1% comercial glucose, 0.8% acid hydrolyzed casein, 0.3% yeast hydrolyte, 0.5% sodium chloride, and 0.1% calcium carbonate. The edium was adjusted to pH values ranging from 6.0 to 8.0, but the itial pH had no effect on viomycin titres. In 500-ml Erlenmeyer sks, 100 ml of the medium, after inoculation with a spore susnsion, was incubated at 24° to 26°C, while being swirled on a tary shaker making 160 revolutions per minute. Antibiotic forman began after 30 hours and rose rapidly to about 600 μg per ml alculated as viomycin sulfate) after 66 hours of incubation. Little ditional antibiotic was formed after this time, but apparently the mycin was not destroyed even after 144 hours of incubation. espective of the initial pH, the medium reached a pH of about in 24 hours, fell slightly to about 6.8 at 48 hours, reached a peak 8.5 at 85 hours, and then remained virtually constant to the end the incubation, i.e., 144 hours. No data are given in this report mycelial growth or changes in the glucose content and nitrogen components of the medium. The titres are good for a first report, but it is reasonable to assume that higher yields have been obtained by the time the fermentation reached the tank-production stage. Production of other antibiotics simultaneously with viomycin has not been reported, but this may be assumed to occur since *Streptomyces* cultures usually form more than one antibiotic.

Crystalline viomycin sulfate has been obtained by adsorption on and elution from carbon followed by successive precipitations from 50% methanol.

CHEMICAL NATURE[23,103,128d]

A tentative empirical formula, $C_{18}H_{31-33}N_9O_8$, has been proposed for viomycin free base. It is a strong organic base that form crystalline salts, e.g., sulfate, hydrochloride, picrate, and reineckate It is of polypeptide nature, contains one primary amino group, bu no free ∝-amino carboxyl. It gives positive Sakaguchi, biuret, an ninhydrin tests, but negative tests for carbohydrate components The ultraviolet absorption spectrum shows a strong maximu which shifts from 268 mμ in 0.1 N HCl to 282.5 in 0.1 N NaOH Viomycin sulfate is levorotatory, but the specific rotation varie from —19.6° to —40.6°, depending on the method of drying th sample and the pH of the solution. Viomycin is stable at roo temperature in acid or neutral solutions for 24 hours or longe At 100°C and alkaline pH, it rapidly loses its antibiotic potency.

After acid hydrolysis and paper chromatography, five maj and three minor ninhydrin spots were found. By passage throug ion-exchange columns four of these ninhydrin positive componen were isolated and three of them identified. These are L-serin ∝,β-diaminopropionic acid, and β-lysine. The fourth compone is a guanidino compound (strong Sakaguchi test) that has bee obtained as a crystalline derivative of *p*-hydroxyazobenzenesulfonic acid, but not characterized further. In addition to t amino acids, hydrolysis of viomycin liberates carbon dioxide, a monia, and urea. The occurrence of two such unusual componen as diaminopropionic acid and β-lysine illustrates the unique cha acter of polypeptide antibiotics.

ACTIVITY[4a,10c,94,103,136,151a,209a,245a]

Viomycin is strongly active against the tuberculosis organis has only low activity against Gram-positive or Gram-negative b teria, and shows no inhibitory effect on fungi. Resistance to vion is acquired by the tubercle bacillus about as readily as stance to streptomycin, but bacteria that have become resistant treptomycin and isoniazid are still sensitive to viomycin. As in case of streptomycin, emergence of strains resistant to viomycin uch retarded when it is given together with another therapeutic ıt, e.g., *p*-aminosalicylic acid. However, since viomycin is less tive and apparently more toxic than streptomycin, it is valuable n adjunct to streptomycin therapy rather than as the agent of choice.

Viomycin is best administered intramuscularly, 2 g or less a day, wo divided doses at 12-hour intervals, twice weekly.

XICITY[10c,128c,203,209a,235c,292]

The acute LD_{50} for mice is given as 241 mg per kg by intra- ous administration and 1,381 mg by subcutaneous route. Elec- yte imbalance, disturbance in renal function, eosinophilia, iness, and deafness have been noted rather frequently in the tment of human patients, but these symptoms are usually mild f short duration. However, as the drug has not been extensively d, clinicians advise that careful attention should be given to ds of the individual patient, size of dose, period of treatment, development of unfavorable symptoms.

COMPARISON OF COMMERCIAL ANTIBIOTICS

Comparison of the potencies of different commercial antibiotics inst specific organisms cannot be made with any assurance from early reports, because different investigators used different hods of assay (e.g., cup, agar streak, dilution, turbidity, etc.) also because they used different strains of the same organisms. h miscellaneous data have a very limited value for purposes of parison. It is also desirable to test the antibiotics against or- isms freshly isolated from human infections and against stock ures in laboratory collections.

Recently more useful data have appeared in the literature. reported in these papers,[37,99,100,101,142,143,165,195,197e,225a,285,288,299] ral commercial antibiotics have been tested simultaneously nst selected organisms under identical conditions by a single ıp of investigators. These tests show that some antibiotics are ıitely more potent than others against certain pathogens. In r papers, many strains, both laboratory stocks and new isolates, been tested against several antibiotics in order to discover the variations among the strains. The variations are often great and indicate that types made resistant by previous exposure to the antibiotics are appearing.

Continuation of research along these lines should be of great value to the clinician who is trying to select the most suitable antibiotic for treatment of a particular disease.

NONTHERAPEUTIC USES FOR ANTIBIOTICS

A recent and unexpected development in the field of antibiotics is the stimulatory effect these substances have on the growth of young animals. This effect was first noted by Moore et al.[156a] and has since been reported by many investigators.[9,37d,47,56,65a,73,124,143a, 156g,170a,171,214b,231a,232a,242a,248,249a,250,297] When fed in low concentration antibiotics usually increase the growth of chicks, pigs, and calve by 10 to 20% in the first few weeks of life and in some experiment the increase in weight has been even greater. Utilization of th ration also appears to be more efficient in the presence of antibiotics The mortality of young animals is also usually much reduced an the weak and unthrifty individuals seem to benefit most from th added antibiotics. As a result of these experiments, antibiotics ar usually added to mixed feeds, particularly to those for poultry Concentrates are manufactured by the antibiotics producer and sol to the mixed feed dealers, who incorporate these into the feed t give an antibiotic content of 1 to 10 g per ton of feed. There ar many such concentrates on sale under various trade names. Th tonnage of mixed feeds for poultry and swine in the United Stat is about 25 million tons a year. Much of this is now supplemente with antibiotics, so it is evident that the outlet for antibiotics i this field must amount to millions of dollars annually.

It is not clear how antibiotics act to produce the increase growth of animals, but the weight of evidence is in favor of the vie that they eliminate harmful bacteria or reduce the number microorganisms in the intestinal tract that compete with the anim for essential nutrients. Strong support for this interpretation found in the evidence that germ-free animals give no response to th addition of antibiotics to the ration.

The effect of antibiotics on the growth of plants and contr of plant diseases by means of antibiotics is being actively investigat and encouraging results have been obtained. The most wide tested antibiotics are Actidione for fungal diseases and streptomyc ant diseases of bacterial origin. The literature is now quite sive and several reviews on it have been written.[7b,163b,197a,286a] creased growth of plants under aseptic conditions in the pres- of certain antibiotics, *e.g.*, penicillin and Terramycin, is an un- ted and puzzling result.[197b]

)ther nontherapeutic uses of antibiotics, such as the preserva- of certain foods and control of industrial infections, are sub- of current research.[235d]

NONCOMMERCIAL ANTIBIOTICS

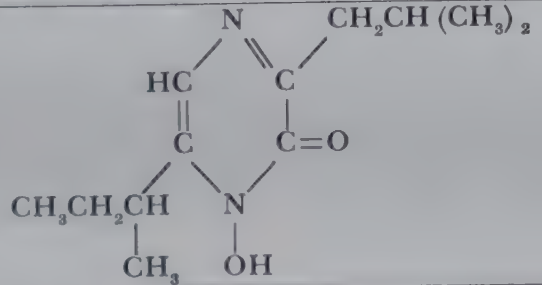
n addition to the antibiotics that have been discussed in detail e preceding sections of this chapter, there are many interesting vell-defined antibiotics which because they appear to lack com- al possibilities do not warrant extended discussion here. How- a summary of important facts about some of these antibiotics een made and this information is given in Table 36. In mak- his selection, only antibiotics that are fairly well defined both a chemical and a biological point of view have been included. ers may disagree with the selections as being too inclusive or xclusive. If they have erred, the authors are inclined to think the fault has been in the direction of too extensive a list rather one that is too limited.

n compiling this table, it has not been possible, unfortunately, ve complete information on the individual compounds. Many rtant facts have had to be omitted because of space limitations sed by a tabular arrangement. It is believed that the con- ess and convenience of the table arrangement is to be preferred descriptive statement concerning each antibiotic.

n selecting the references, an effort has been made to include rst mention of the antibiotic, papers dealing with information in the several columns, and the last paper on the compound ved at the time the table was compiled.

Table 36 indicates that the actinomycetes and the fungi are nief sources of antibiotics. In recent years, the trend has been strongly in the direction of the actinomycetes than toward the sources. Bacteria do not play a very prominent part in the otic program. Most of the antibiotics produced by bacteria lypeptides and so far none of these has proved to be safe for teral use. Restriction to topical use has placed a severe limita- n their value.

Table 36. SUMMARY OF MAIN FACTS ABOUT OTHER WELL-DEFINED ANTIBIOTICS

Antibiotic	Source	Formula or chemical nature	Activity[a]	Toxicity, LD_{50}[b] mg per kg	References
Actinomycin A, B, C	*Actinomyces antibioticus,* etc.	Chromopeptides. A and B yield L-methylvaline, D-valine, sarcosine, L-proline and L-threonine; quinone (?). C gives D-isoleucine or D-alloisoleucine in place of D-valine.	G+ Y G− Y (?) F Y	0.5 (Ip or Sc)	48, 74, 157, 272, 279, 280
Actinorubin	*Actinomyces* strain A105	$C_6H_{14}O_2N_3$ or $C_9H_{22}O_4N_5$ (?)	G+ Y G− Y	36 (Ip)	149, 156, 187
Antimycin A	*Streptomyces* (unidentified)	$C_{28}H_{40}O_9N_2$. Yields: L-threonine, 3-aminosalicylic acid, L-methylethylacetic acid, and a $C_{11}H_{20}O_3$ acid.	F Y	25 (Sc)	2, 85, 156b, 164, 233b, 257a
Aspergillic acid	*Aspergillus flavus*		G+ Y G− Y F Y My Y	48.5 (Iv)	84, 86, 170, 204a, 219, 296, 302
Aureothricin (cf. thiolutin)	*Aspergillus farcinicus*	$C_9H_{10}N_2O_2S_2$. Yellow crystals. It is the propionamido derivative of the base $C_6H_6N_2OS_2$	G+ Y G− Y My Y	500 (Sc)	61a, 173, 262
Ayfivin (same as bacitracin A, B, and C)	*Bacillus licheniformis* strain A-5	A mixture of polypeptides. Yields aspartic acid, glutamic acid, histidine, ornithine, lysine, cystine, phenylalanine, leucine and isoleucine	G+ Y G− Slight	ca 500 (Sc)	15, 16, 132a, 197, 236
Borrelidin	*Streptomyces rochei*	$C_{28}H_{43}O_6N$ An acid with conjugated double bonds. Yields crystalline methyl and *p*-nitrobenzyl esters and other derivatives	*Sarcina lutea* Y Most bacteria N *Borellia* Y Enhances penicillin	74.7 (Sc) 39.0 (Iv)	29, 53, 126, 233c

[a] [illegible] Gram-positive bacteria = G+, Gram-negative bacteria = G−, filamentous fungi = F, mycobacteria = My;

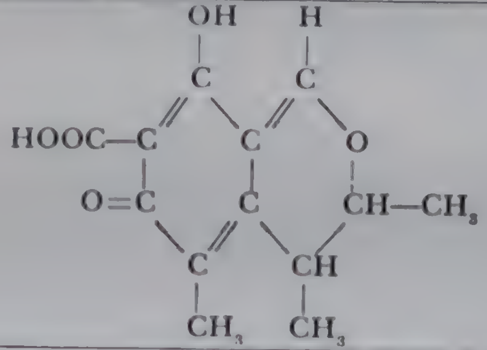
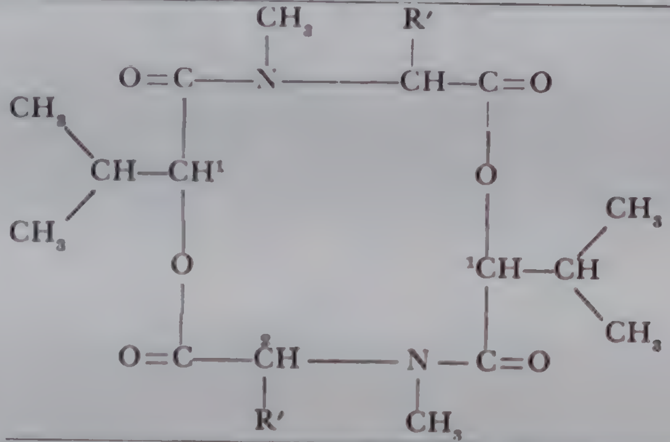
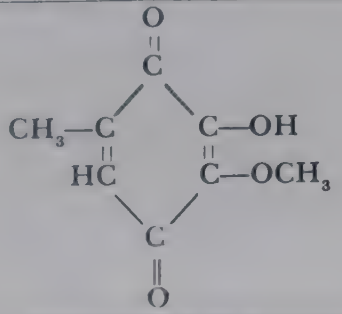
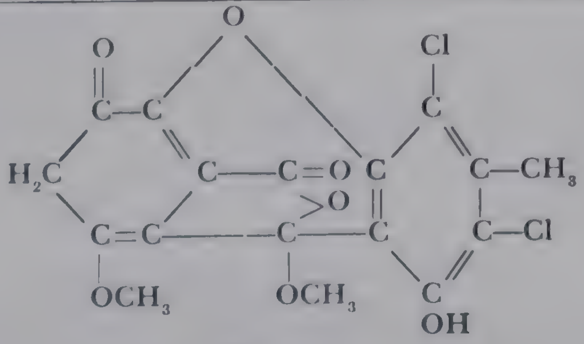
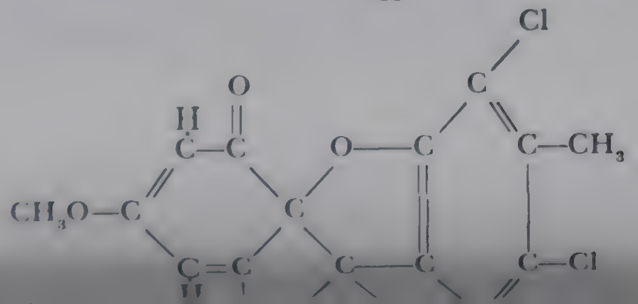
Chetomin	*Chaetomium cochliodes,* etc.	$C_{10}H_{17}O_4N_3S_2$. Contains indole, four active hydrogens, methylimide and other groups, but no basic or acidic groups	G+ Y G– Y	"not very toxic"	117, 118, 269
Citrinin	*Penicillium citrinen,* etc. *Aspergillus niveus*		G+ Y G– N My (?) Protozoa Y	35 (Sc, Ip)	4b. 60, 71, 121, 127a, 147a, 216, 28
Enniatin (A, B, C) (Lateritiin, avenacin, fructigenin, sambucinin)	*Fusarium orthoceras* var. *enniatinum* etc.	 In Enniatin A: $R' = CH(CH_3)C_2H_5$ In Enniatin B: $R' = CH(CH_3)_2$ In Enniatin C: $R' = CH_2CH(CH_3)_2$ [1] D-(−)-α-hydroxyisolvaleric acid 2 mols [2] N-methyl-L-isoleucine 2 mols (A), or *N*-methyl-L-valine 2 mols (B), or *N*-methyl-L-leucine 2 mols (C)	G+ Y G– Y	"Low"	67, 114, 210

TABLE 36 (Continued)

Antibiotic	Source	Formula or chemical nature	Activity[a]	Toxicity, LD_{50}[b] mg per kg	References
Fumigacin (Helvolic acid)	*Aspergillus fumigatus*	$C_{32}H_{44}O_8$ (?) (A keto monobasic acid)	G+ Y G− N	400 (Ip)	35, 63, 95, 185, 274
Fumigatin	*Aspergillus fumigatus*		G+ Y G− N		11, 199, 271



or



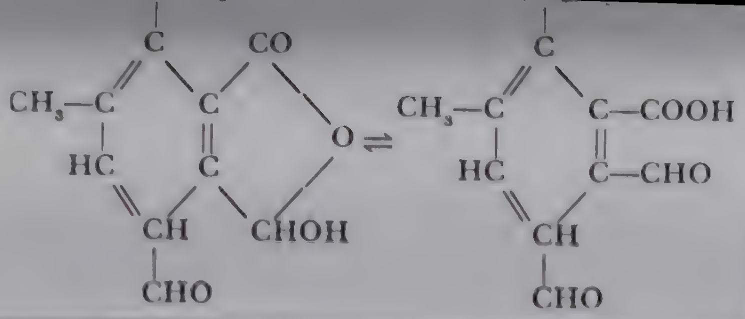
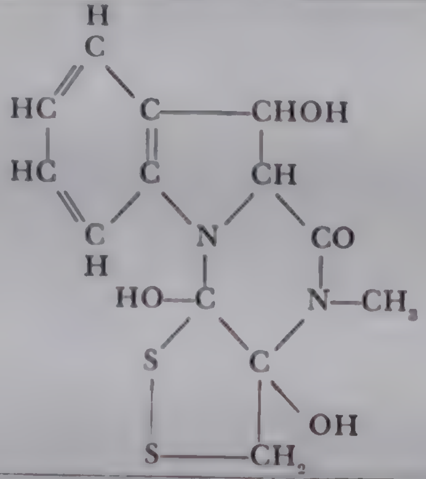
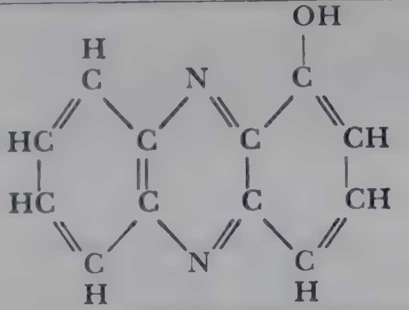
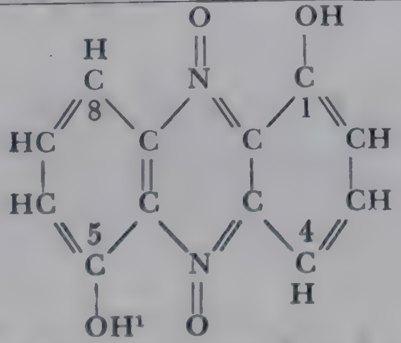
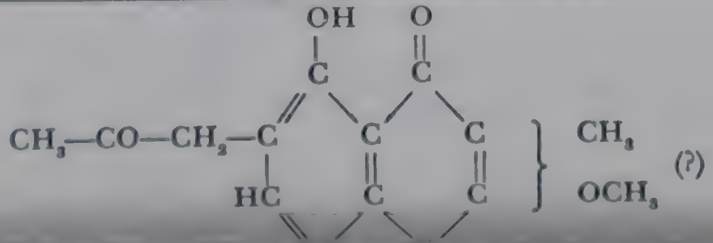
Gladiolic acid	*Penicillium gladioli*		G+ Y G− N F Y		39, 41, 124a, 182
Gliotoxin	*Trichoderma lignorum (viride)* Aspergilli and penicillia		G+ Y G− N F Y My Y	19.5 (Iv)	38, 43, 88, 96, 147b, 156a, 185, 219, 233, 281, 286
Glutinosin	*Metarrhizium glutinosum*	$C_{48}H_{60}O_{16}$	G+ N (?) G− N F Y		40, 44, 46
Glyco-lipide	*Pseudomonas aeruginosa*	$C_{32}H_{58}O_{13} \cdot H_2O$ A conjugate of L-rhamnose (2 mols) and L-β-hydroxydecanoic acid (2 mols)	My Y	5 mg per mouse kills in 16 hours (Ip)	145
Griseolutein	*S. griseoluteus*	$C_{17}H_{15}O_6N_3$	G+ Y G− Y My Y	> 2000 (Sc)	197d, 259, 307a

TABLE 36 (*Continued*)

Antibiotic	Source	Formula or chemical nature	Activity[a]	Toxicity, LD_{50}[b] mg per kg	References
α-Hydroxyphenazine (hemipyocyanine)	*Pseudomonas pyocyanea* (aeruginosa)		G+ Y (?) G− Y (?) F Y	Less toxic than pyocyanine	234, 249
Illudins M and S	*Clitocybe illudens*	$C_{15}H_{20}O_3$, M $C_{15}H_{22}O_4$, S	G+ Y G− Y My Y F Y	$LD_{100} = 15.6$	6
Iodinin	*Chromobacterium iodinium*	 [1] OH is at either 5 or 8	G+ Y G− Slight		65, 181
					

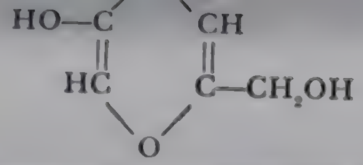
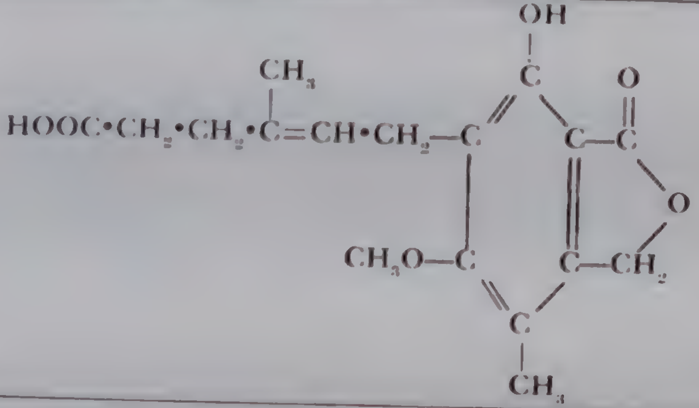
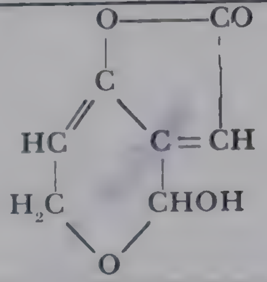
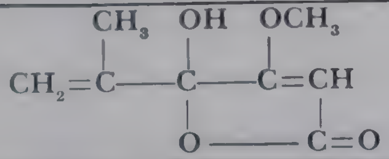
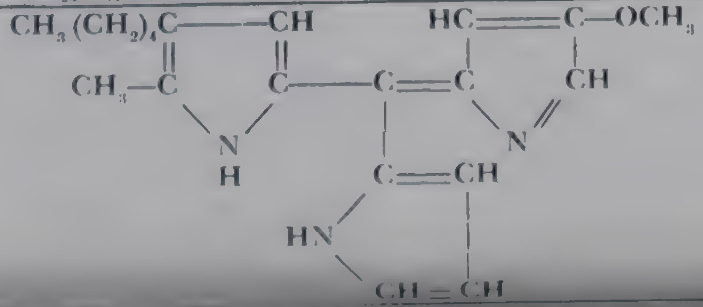
Kojic acid	*Aspergillus flavus*, etc.		G+ Y (low) G− Y (low)	120-180 (Ip)	68, 146, 189
Lavendulin	Organism resembling *Streptomyces lavendulae*	$C_{49}H_{83}O_{18}N_{13}S_{3}$ (?) Lavendulin-helianthate	G+ Y G− Y	28 (Ip) 14 (Iv)	149, 156 187
Licheniformin	*Bacillus licheniformis*	Mixture of cyclic peptides. M.W. 3,800-4,000. Vary in potency and activity. Yield common amino acids on hydrolysis	G+ Y G− Y My Y	250 (Iv) 375 (Ip) 500 (Sc)	55
Marasmic acid	*Marasmius conigenus*	$C_{18}H_{20}O_4$ Unsaturated monobasic acid	G+ Y G− N F (?)	16-32 (Iv)	155
Mycophenolic acid	*Penicillium brevi-compactum*, etc.		G+ Y G− N F Y	$LD_{100} = 500$ (Iv)	1a, 34, 36a, 106
Mycosubtilin	*Bacillus subtilis* 370	Polypeptide containing aspartic acid, tyrosine, and proline. Other amino acids uncertain.	G+ N (?) G− N F Y	25 lethal 12.5 not lethal (Sc)	284
Netropsin	*Streptomyces netropsis*	$C_{32}H_{48}O_4N_{18}\cdot 4HCl$. Gives guanidine base, $C_8H_5ON_3$, and monobasic acid, $C_{15}H_{20}O_3N_6$	G+ Y G− Y F Y	17 (Iv) 70 (Sc)	102

TABLE 36 (*Continued*)

Antibiotic	Source	Formula or chemical nature	Activity[a]	Toxicity, LD_{50}[b] mg per kg	References
Nisin	*Streptococcus lactis*, M354/07	Mixture of polypeptides. On hydrolysis gives lanthionine, cystathionine (or allocys.) and common amino acids. Cf. cinnamycin and subtilin	G+ Y	Between 20-30 (Iv, rabbit)	32, 33, 33a, 122a, 133, 134, 179
Patulin (clavacin clavatin claviformin expansin)	*Penicillium patulin*, etc. *Aspergillus clavatus*, etc.		G+ Y G− Y F Y My Y	20-25 (Iv, Sc) 10 (Ip)	12, 28, 62, 74a, 146a, 193, 213, 266, 273, 305
Penicillic acid	*Penicillium puberulum*, etc. *Aspergillus ochraceus*		G+ Y G− Y My Y	110 (Sc) 250 (Iv)	4, 35a, 107, 190, 200, 221
Pleurotin	*Pleurotus griseus*	$C_{20}H_{22}O_5$	G+ Y G− N F Y	> 24 (Iv)	154, 229
Proactinomycin A, B, C	*Proactinomyces (Nocardia) gardneri*	A: $C_{27}H_{47}O_8N$ B: $C_{28}H_{49}O_8N$ C: $C_{24}H_{41}O_6N$	G+ Y G− (?)	A: 150 (Iv) B: 120 (Iv) C: 80 (Iv)	112, 176, 177
	Serratia marcescens		G+ Y (?) G− Y (?) F Y P Y		18a, 105, 160

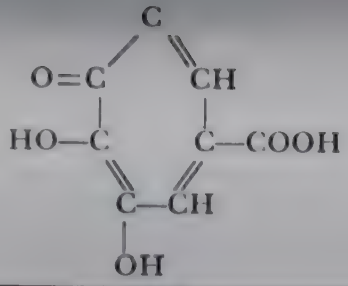
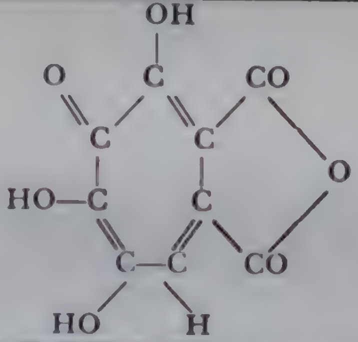
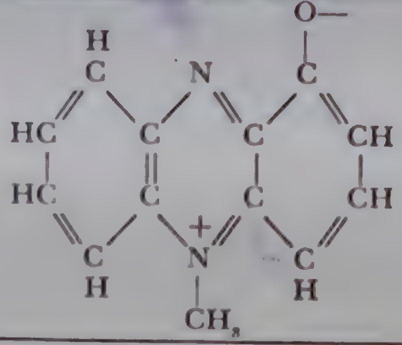
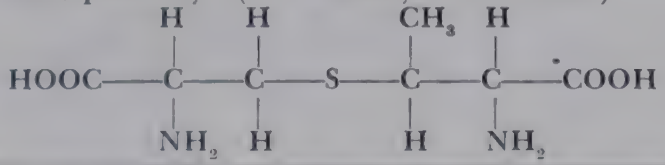
Puberulic acid	*Penicillium puberulum,* etc.		G+ Y G− N		19, 36, 69, 182
Puberulonic acid	*Penicillium aurantiovirens,* etc.		G+ Y G− N		16b, 19, 36, 69, 146b, 182
Pyo compounds	*Pseudomonas aeruginosa*	I_b, $C_{16}H_{21}NO$, 2-heptyl-4-quinolinol I_c, $C_{18}H_{25}NO$, 2-nonyl-4-quinolinol II, $C_{17}H_{23}NO_2$, ? III, $C_{18}H_{23}NO$, 2-(△′-nonenyl-) -4-quinolinol IV, $C_{16}H_{23}NO_3$, ?	G+ Y G− Slight	In carbonate 25-38 In fat about 75 (Ip)	129, 289a, 290, 291
Pyocyanine	*Pseudomonas aeruginosa,* etc.		G+ Y G− YN F N	MLD is 100	198, 234, 249

TABLE 36 (*Continued*)

Antibiotic	Source	Formula or chemical nature	Activity[a]	Toxicity, LD_{50}[b] mg per kg	References
Pyolipic acid	*Pseudomonas pyocyanea*	A conjugate of L-β-hydroxydecanoic acid ($C_7H_{15}CHOH \cdot CH_2 \cdot COOH$) and L-rhamnose ($CH_3(CHOH)_4CHO$)	My Y		30, 31
Streptolin	*Streptomyces* (unidentified)	$C_{17}H_{31}O_8N_5 \cdot 3HX$ or $C_{24}H_{45}O_{11}N_7 \cdot 4HX$ Gives β-lysine,* and two other ninhydrin-positive compounds on hydrolysis.	G+ Y G− Y	5 (Iv)	161, 208, 228, 237a, 266a
Streptothricin	*Streptomyces lavendulae*	$C_{20}H_{34}O_9N_8 \cdot 3HX$ (hydrochloride, etc.) On hydrolysis gives β-lysine* and two other ninhydrin-positive products.	G+ Y G− Y My Y F Y	75 (Iv)	59, 109, 111, 141, 205, 218, 230, 252, 268, 301
Subtilin	*Bacillus subtilis*	Polypeptide. N.W. about 7,000. Yields eleven common amino acids, lanthionine and a new S-amino acid, probably: (Cf. cinnamycin and nisin)  $\begin{array}{ccccccccccc} & & H & & H & & & & CH_3 & & H \\ & & \vert & & \vert & & & & \vert & & \vert \\ HOOC & — & C & — & C & — & S & — & C & — & C — \dot{C}OOH \\ & & \vert & & \vert & & & & \vert & & \vert \\ & & NH_2 & & H & & & & H & & NH_2 \end{array}$	G+ Y G− Y My Y Viruses A&B of influenza	670 (Sc)	3a, 3b, 7, 47a, 56b, 97, 113, 144, 167, 168, 232, 251
Sulfactin	*Actinomyces* Sp. (possibly *roseus*)	$C_{38}H_{55}O_7N_{11}S_4$ or $C_{27}H_{40}O_5N_8S_3$	G+ Y G− N	149 (Ip)	150, 188
Thiolutin (cf. aureothricin)	*Streptomyces albus*	$C_8H_8N_2O_2S_2$. Yellow-orange neutral crystals. Acid hydrolysis gives acetic acid and a monoamine, $C_6H_6N_2OS_2$.	G+ Y G− Y My Y F Y P Y	25 (Sc)	61a, 235b, 257

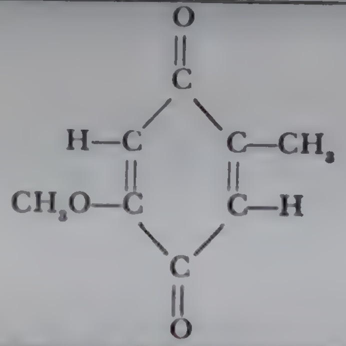
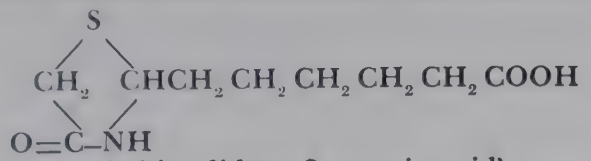
Toluquinone (5-methoxy-p-toluquinone)	*Coprinus simulus* *Lentinus degner*		G+ Y G− Y F Y	25 (Iv)	5
Ustin, I, II, III	*Aspergillus ustus*	I, $C_{21}H_{17}O_6Cl_3$ II, $C_{21}H_{14}O_6Cl_2$ III, $C_{19}H_{15}O_5Cl_3$	G+ Y G− N My Y	6-8 mg tolerated by mice	77, 138, 216a
Viridin	*Trichoderma viride*	$C_{19}H_{16}O_6$. Contains one methoxy group. Two components are isomeric, but differ in properties. Very active against fungi.	G+ N G− N F Y		42, 45, 182, 267a
Xanthomycin	*Streptomyces* (unidentified)	$C_{23}H_{30}O_7N_3 \cdot 2HX$. It is a methyloxylated quinone joined to a basic moiety. Gives methylamine, ethanolamine, etc., on degradation.	G+ Y G− Y	0.16 (Iv)	186, 220a, 258

TABLE 37. SUPPLEMENTARY TABLE OF WELL-DEFINED ANTIBIOTICS*

Antibotic	Source	Chemical Nature	Activity**	Toxicity,† LD_{50}, mg per kg	References
Actithiazic acid (Mycobacidin)	*Streptomyces virginiae*, *S. cinnamonensis*	 (4-thiazolidone-2-caproic acid)	My Y G+ N G− N	More than 1500 (Iv or Sc)	S17, S28, S36, S43, S46, S55, S56, S59
Alternaric acid	*Alternaria solani*	$C_{21}H_{30}O_8$ (Unsaturated dibasic acid)	F Y (low or no activity for bacteria)	Toxic to some plants	S8, S9, S26, S50a
Amicetin	*Streptomyces* sp.	$C_{29}H_{44}N_6O_9$. Yields cytosine, *p*-aminobenzoic acid, D-α-methyl serine and an unidentified product.	G+ Y G− Y My Y (marked)	90 (Iv) 600-700 (Sc)	S20, S20a, S45a
Bacillomycin	*B. subtilis*	Consists of several peptides. B component gives common amino acids probably high in glutamic.	G+ N G− N F Y	50-75 (Iv)	S40, S60, S65
Candicidin A, B, and C.	*Streptomyces griseus*	A: $C = 62.9\%$; $H = 9.6$; $N = 4.7$ B: $C = 57.8\%$; $H = 9.9$; $N = 7.3$	G+ N G− N My N F Y	Crude 663 A 277 } Sc B 159 Crude 79 A 47 } Iv B 53	S38, S41
Catenulin	*Streptomyces* sp.	$C = 50.2\%$; $H = 5.3$; $N = 10.1$ $S = 7.9$ (sulfate)	My Y	125 (Iv)	S19

* January 1951 to June 1953.

** Y = yes; N = no; G+ = Gram-positive bacteria; G− = Gram-negative bacteria; F = fungi; My = mycobacteria; P = protozoa.

† An asterisk (*) in this column indicates no data found in published papers

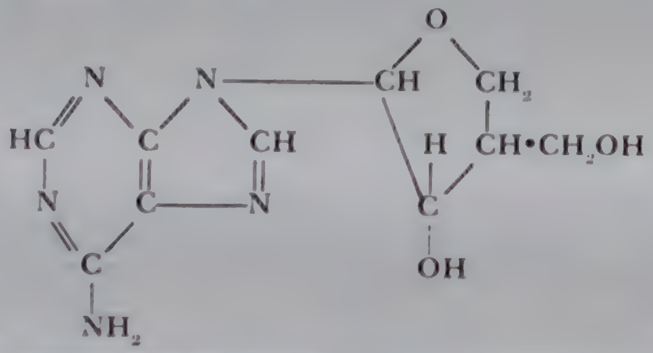
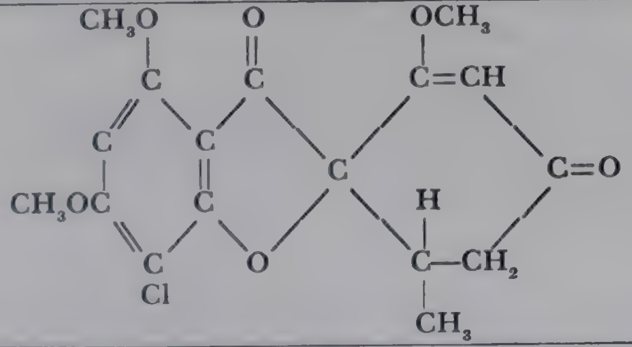
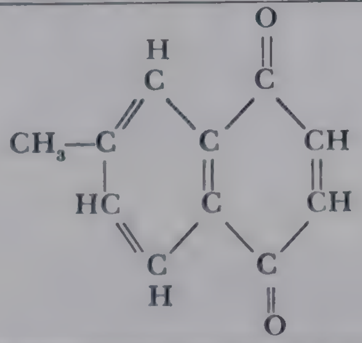
Cephalosporins P_1, P_2, P_3, P_4, P_5, N	*Cephalosporium* unknown species	P_1, P_2, P_4 (acidic) crystallized P_1: $C = 65.8\%$; $H = 9.0$; $C—CH_3 = 12.1$, acetyl $= 16.8$, active $H = 0.6$. P_2: $C = 65.25$; $H = 8.5$; $C—CH_3 = 9.4$, $OCH_3 = 5.8$; acetyl $= 18.8$; active $H = 0.8$.	P: G+ Y G− N N: G+ Y G− Y	P_1, 500 (Iv)	S15, S48a, S53
Cinnamycin	*Streptomyces cinnamoneus*	Polypeptide. Contains common amino acids; also lanthionine and $C_7H_{14}N_2O_4S$ amino acid; cf. nisin and subtilin.	G+ Y My Y	*	S2
Cordycepin	*Cordyceps militaris*		G+ Y My Y	"Of a low order"	S3, S18, S26
Exfoliatin	*Streptomyces exfoliatus*	$C_{27}H_{40}O_{16}Cl \cdot H_2O$ (?)	G+ Y G− N (except hemophilus)	500 (Sc)	S63
Fungistatin (antibiotic XG)	*Bacillus subtilis*	Polypeptide. Gives common amino acids.	F Y	90 (Iv)	S34, S41a, S51
Grisein	*Streptomyces griseus*	Probably $C_{40}H_{61}N_{10}O_{20}SFe$.	G+ Some G− " My " F N	None reported even with large doses	S39, S52

TABLE 37 (*Continued*)

Antibiotic	Source	Chemical Nature	Activity**	Toxicity,† LD_{50}, mg per kg	References
Griseofulvin	*Penicillium griseofulvum*		G+ N G− N F Y	Toxic to some plants	S6, S7, S27, S42, S47a, S49
Luteomycin	*Streptomyces* n. sp.	$C_{25}H_{29}NO_9 \cdot HCl$	G+ Y G− N	6 (Iv or Sc)	S30, S31, S48, S54
6-Methyl-1,4,—naphthoquinone	*Marasmius graminium*		G+ Y	*	S1a
Mycomycin	*Nocardia acidophilus* n. sp.	$HC{=}C{-}C{=}CCH{=}C{=}CHCH{=}CH{-}CH{=}CHCH_2COOH$ 3,5,7,8-tridecatetraene-10,12-diynoic acid.	G+ Y G− Y My Y F Y	Apparently nontoxic	S16, S37
	Streptomyces unidentified	$C_{39}H_{68}O_{11}$ (monobasic acid)	G+ Y G− N My Some	2.5 (Ip)	S29

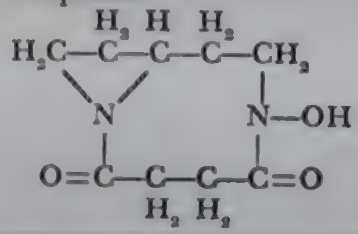
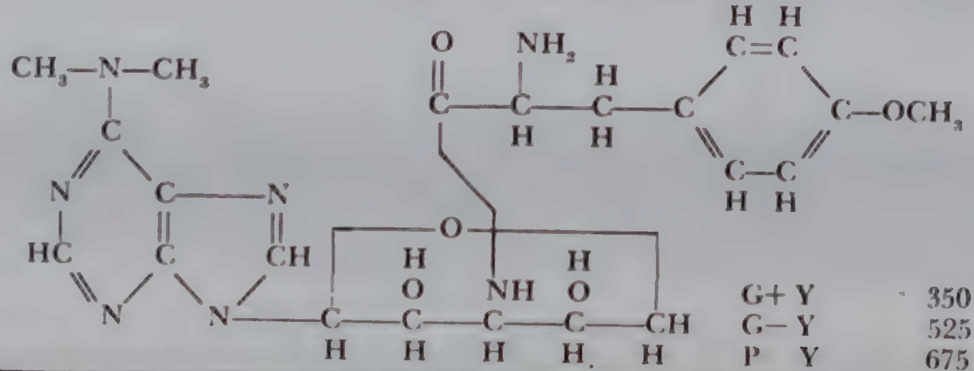
Nitrosporin	*Streptomyces nitrosporeus* n. sp.	$C_{30}H_{26}N_2O_6$ (?)	G+ Y G– Y	16 (Iv)	S62
Nocardamin	*Nocardia*	Proposed: 	G+ "little" G– " My Y		S57, S58
Pikromycin (Cf. Proactinomycin in Table 36)	*Actinomyces* sp.	$C_{25}H_{43}NO_7$ (N present in dimethylamino group)	G+ Y	Lethal dose (Iv) 100-700	S13
Polypeptin	*Bacillus krzemieniewski*	Mixture of basic polypeptides. Main component resembles polymyxius in amino acid content.	G+ Y G– Y F Y	15	S31a, S35, S47
Puromycin	*Streptomyces alboniger*	$C_{22}H_{29}N_7O_5$ 	G+ Y G– Y P Y	350 (Iv) 525 (Ip) 675 (oral)	S1, S50, S64

TABLE 37 (*Continued*)

Antibiotic	Source	Chemical Nature	Activity**	Toxicity,† LD_{50}, mg per kg	References
Resistomycin	*Streptomyces resistomycificus*	$C_{23}H_{18}O_6$ "weakly acid" "4 active H atoms"	G+ Y My Y	•	S14
Rhodomycins (A and B)	*Streptomyces purpurascens*	$C_{20}H_{29}NO_7$ or $C_{40}H_{58}N_2O_{14}$	G+ Y G– slight F N	•	S10, S11, S12
Thioaurin	*Streptomyces* sp.	$C_{14}H_{12}O_4N_4S_4$ (?)	G+ Y G– Y	16 (Iv)	S5
Trichothecin	*Trichothecium roseum* Link	$C_{19}H_{24}O_5$ (an ester)	F Y	(for plant disease only)	S21, S22, S23
Vinactin A, B, C (Cf. viomycin)	*Actinomyces vinaceus*	Peptides. A and B give lysine, serine, alanine, glycine, glutamic acid, aspartic acid. C gives serine, sarcosine, glycine, and an unidentified amino acid.	G+ Y G– Y My Y Rickettsia Y	•	S45, S61
X-206 X-464 X-537A	*Streptomyces* unidentified species	$C_{46-47}H_{80-82}O_{13}$ $C_{25}H_{40}O_7$ $C_{34}H_{52}O_8$ Optically active organic acids	All alike G+ Y G– N F N	11 (Sc) 2.5 (Ip) 40 (Ip)	S4

In spite of the fact that only antibiotics for which there are derable chemical data have been included in Table 36, a study ese data shows that there is a dearth of knowledge on the ical nature of these substances. Structural formulas are known ss than half of the compounds. It is difficult, costly, and time uming to work out the structure of antibiotics, but the picture not be clear until the structure is known. Only then can a er understanding of the chemotherapeutic action of the anti- cs be obtained. The chemical structures that are known are so d that only a beginning can be made toward developing a ry as to the type of structure a compound must have in order ow biological activity. The mode of action of antibiotics is ably more obscure even than their structural specificity. In r field there is abundant opportunity for fundamental work.

A related and equally obscure feature of antibiotics is the on for their toxicity. The LD_{50} figures range from 0.16 mg per or xanthomycin to several hundred times that figure for a num- of the others. It is not unthinkable that some of the compounds regarded as much too toxic may prove useful against certain tions when administered under carefully controlled conditions. relative nontoxicity of penicillin has led the medical profession pect effective antibiotics of low toxicity. Such compounds are and it may be worth while to go back and restudy some of those have been discarded as well as to continue the search for new pounds with antibiotic activity.

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PART VI. MISCELLANEOUS

CHAPTER 10

MISCELLANEOUS FERMENTATIONS

Alfred R. Stanley and R. J. Hickey

DEXTRAN FERMENTATION

Dextran is a microbiologically produced polysaccharide well known around sugar refineries and present in very small amounts in practically all commercial sucrose. This compound has been used on a limited scale for some time as a stabilizer in certain food products, such as sirups and ice creams, and has recently attained a leading position in the field of blood extenders for use in place of blood plasma in cases of shock and loss of blood.

Historical

As recorded by Browne,[10] the first report on dextran goes back to 1822. In this year, Vanquelin sent four bottles of cane juice from Martinique to France, but on arrival, it was found that the juice had changed to a thick mucilage. Vanquelin studied this gummy mass, but could not determine its composition, probably because of a lack of analytical procedures. Pasteur, Durin, Scheibler, and many others studied and described this and similar gummy fermentations of both cane and beet sugar and, in 1861, Pasteur[69] described two "sorts" of viscous fermentations due to two di "organized ferments."

Durin,[17,18] was convinced that this gum was composed of lose, but its true composition, consisting only of glucose mole was first discovered by Scheibler.[79] He named it dextran beca was similar to dextrin, but was in error in believing the substa occur naturally in cells of the sugar beet.

The causal organism of this fermentation was first nam Cienkowski[15] as *Ascococcus mesenteroides*, believing the sli be due to a true zoogleal formation. Van Tieghem[107] gave th adequate description of the organism noting its similarity t genus *Nostoc* of the green algae and so he named it *Leuco* *mesenteroides.*

Several other workers, including Bechamp,[6] Daumic Brautigam,[9] and Happ[38] studied the fermentation from di points of view, but with no particularly outstanding results.

Pure cultures of *Leuconostoc mesenteroides* were first is by Leisenberg and Zopf[58] and strains from different sources s no marked variation. Two other isolants were named *L. ope* and *L. aller* by Zettnow.[118] Another strain named *Lacto* *dextranicus* was isolated by Beijerinck[7] in 1914. It was descri a lactic acid-producing coccus which formed dextran fron sucrose.

A very careful study of the genus *Leuconostoc* was con by Hucker and Pederson[45] who classified three distinct s namely *L. mesenteroides*, *L. dextranicus*, and *L. citrovorus* classification is based primarily on reactions in a variety of hydrates and polyhydric alcohols. From this classification, it i certain that the organisms described by Liesenberg and Zop by Zettnow[118] were of the species *mesenteroides*, whereas th scribed by Beijerinck and later by Fernbach, Schoen, and wara[25,26] were of the *dextranicus* type.

More detailed reviews covering the period up to the ear of this century are those of Lafar,[57] from the microbiologica of view, and of Lippmann,[58] from the viewpoint of a sugar c

Raw Materials

Sucrose is the principal raw material for dextran for It may be used in the form of molasses, as noted by Bau rn,[88] or as refined sucrose with which most of the work has been e. In addition, plant juices have served as the raw material in eral studies, for example, aquamiel,[72] sugar-beet juice,[2,102] e,[100] and sugar-cane juice.[114] Most investigators report that rose is the only carbohydrate from which they were able to ob dextran, but Leuenberg and Zopf[51] reported the production of tran from both sucrose and dextrose. The work of the last 29 rs does not, however, tend to substantiate this.[88]

In 1931, Tarr and Hibbert[109] reported that, with four strains *L. mesenteroides* studied, pronounced precipitation occurred in mented sucrose media treated with 5 volumes of 95% alcohol. ly slight precipitation was found in glucose media with the two ains received from Holland when treated in the same manner. ey conclude from this that "It seems probable that only very ive cultures of *L. mesenteroides* are capable of forming dextran m glucose."

The production of dextran from refined cane sugar has been own to be stimulated by certain crude plant products, including sugar,[11] maple sugar,[80] and an extract of figs.[108]

The conversion of dextrin, Lintner's soluble starch, certain drolysates of amylopectin, and of crystalline amylose into dextran been described.[60a,61] The acetic acid bacteria are involved in this version, whereas the lactic acid types are used with sucrose.

Fermentation Mechanism

The mechanism of the fermentation producing dextran from rose is not too well understood.

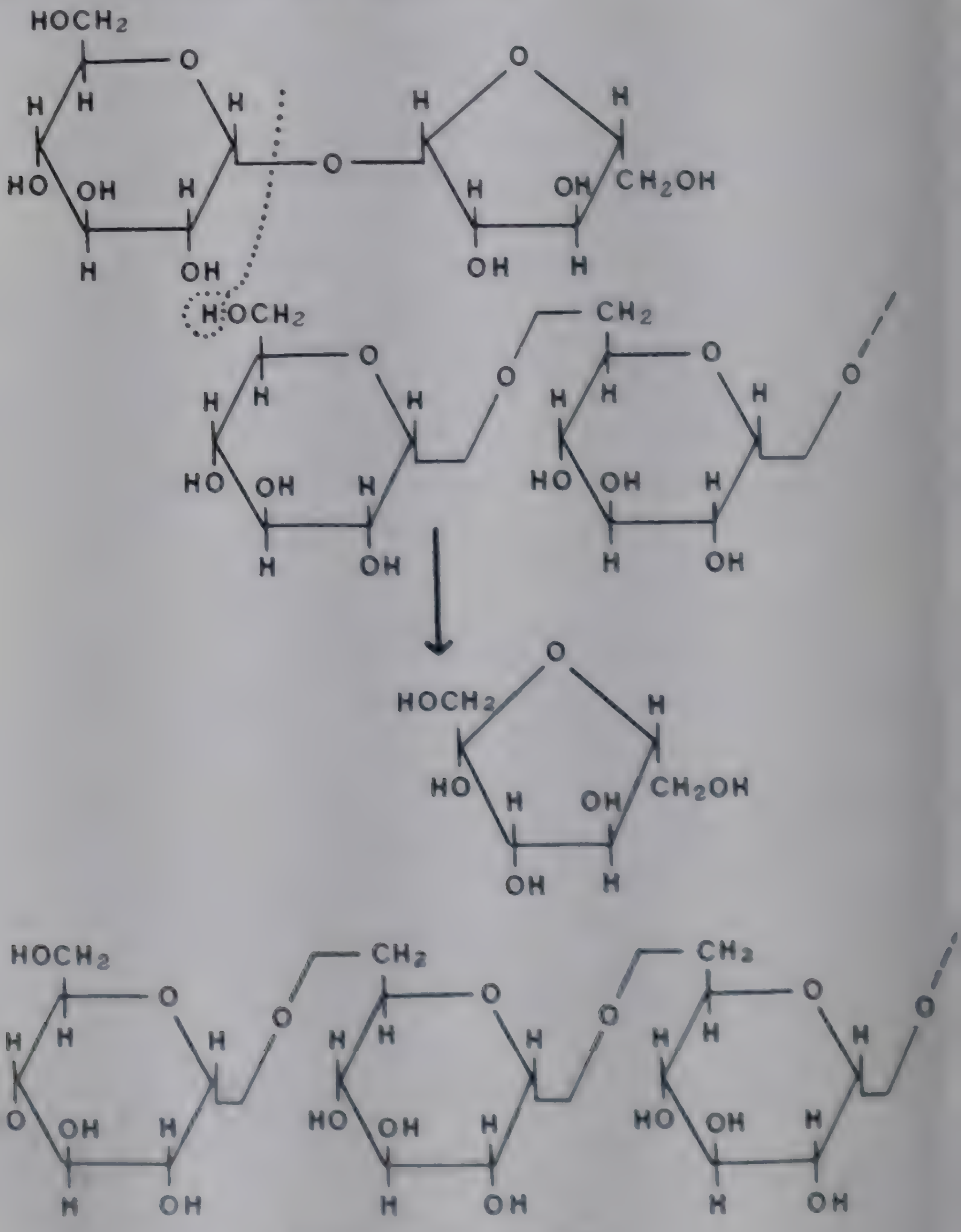
Kagan, Lyatker, and Tsvasman,[46] in 1942, stated that *L. mesen-oides* was capable of binding inorganic phosphorus in the pres e of sucrose, but not of glucose or fructose. In sucrose, then, the ri ester is formed, as is shown by its accumulation at the expense inorganic phosphorus. Later, Hehre[29,30] concluded from his dies that phosphorylated sugar does not enter into this synthesis dextran.

Leibowitz and Hestrin[52] stated in 1945 that the Cori ester s not enter into this reaction nor is sucrose acted on by the or ary phosphorylase. They concluded that, while the exact mecha m is not certain, the process is direct and involves a primary rolysis.

In 1950, Hassid and Doudoroff[35] stated that the reaction i volves the substitution of a 1,6-glucosidic linkage for the glucos fructose bond in the sucrose molecule. Evidently only the gluco portion of sucrose is used for formation of the dextran molecul Neither glucose alone nor glucose-fructose mixtures will yield de tran. Sucrose is considered essential.[8a]

A reaction for the formation of dextran from sucrose by th action of dextransucrase appears to be as follows:[40a]

In addition to the 1,6-glucosidic linkage in dextran, some 1, maltosidic cross-linkage is also found.[8a,40b]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(adapted from Hehre[40a])

Cultures and Media

Several different media and modifications have been described use with *Leuconostoc mesenteroides.* Tarr and Hibbert,[101] in 31, suggested 10% sucrose, 0.5% KH_2PO_4, 0.5% NaCl, and 0.5% ptone. A recent report[86] indicates that *L. mesenteroides* NRRL 512 is generally employed for the preparation of clinical dextran commerce.

The use of maple sugar as a part of the sucrose was suggested[80] 1938. This undoubtedly contributed a number of growth factors.

Yeast extract was substituted for peptone, and ammonium and agnesium sulfates were added by Hassid and Barker,[34] in 1940. heir medium was as follows: 600 g sucrose, 15.0 g yeast extract, g K_2HPO_4, 1.2 g $MgSO_4 \cdot 7H_2O$, 3.6 g $(NH_4)_2SO_4$, and 6 l distilled ater.

This medium was modified by Jeanes, Wilham and Miers[48] who lded sodium chloride in 0.1% concentration. Other variations e composed of the previously listed ingredients, but in different roportions. Calcium carbonate has sometimes been added to edia to control the pH.[48]

Suggested substitutes for peptone and yeast extract are corn eep liquor,[68,111] malt sprouts,[111] proteolyzed wheat grain,[68] and ydrolyzed juice of *Agave atrovireus.*[72]

Stacey and Swift,[76] in 1948, described a somewhat different mbination as follows: 4.9 g microcosmic salt, 0.5 g *p*-aminobenzoic id, 1.0 g potassium hydrogen phosphate, 0.5 g potassium chloride, 5 g hydrated magnesium sulfate, 0.01 g hydrated ferrous sulfate, 01 g peptone, 100 g sucrose, and 1,000 ml distilled water.

The production of dextran by other bacteria was reported by ehre and coworkers.[41,42] When growing *Acetobacter viscosum A. capsulatum,* they used a medium consisting of 5% dextrin or her carbohydrate and 0.5% yeast extract, while the broth for reptococci contained 5% sucrose, 1% tryptose peptone, 0.2% hydrous sodium phosphate, and 0.5% sodium chloride.

Incubation temperatures have varied somewhat between investigators. *Leuconostoc* strains have been grown at either 20° to 25°C at 30°C and until 1948 the fermentation period was extended r 5 to 10 days. Jeanes, Wilham, and Miers[48] and Sanchez-Marroin and Arciniega[72] reduced this time to between 24 and 26 hours for a high viscosity dextran. *Acetobacter* fermentations were r 25°C for 1 week, whereas the streptococci were incubated at for 5 days.

One of the fungi (*Botrytis cinerea*) has also been desc as producing dextran from sucrose.[109] This organism has some trouble in wine making.

Growth factors required by the various species of *Leuco* have been studied by a number of investigators,[63,74,100] but the studies relating these factors to dextran production are the Carlson and coworkers.[11,12,115,116] They found that all stra *Leuconostoc* studied required thiamine, nicotinic acid, and tothenic acid. Requirements for folic acid and riboflavin with the strains and while biotin was required in a glucose me it was not required in the presence of sucrose. *p*-Aminob acid was not required for dextran synthesis by the strains s and the stimulation afforded by raw cane sugar could not be tified with any of the vitamins, or their combinations, studie

The production of dextran by cell-free enzymes has be scribed by Hehre,[38b,40a] Stacey,[78a] and Koepsell and Tsuc According to the last authors, this enzyme, dextransucrase, produced in large quantity in a corn steep liquor-minera medium. The molecular size of the resulting dextran can be by changing the conditions of the reaction. Large amou fructose are present in the reaction solution when dextran for is completed. Enzymic synthesis of dextran, levan, and othe saccharides was reviewed in 1951 by Hehre.[40a]

Dextran Recovery

It was recognized at least as early as 1903 that dextran c precipitated by alcohol.[71] The proportions used have varie ever, from 1 to 5 volumes as compared with the dextran s

One volume of alcohol per volume of dextran soluti used by Hassid and Barker,[36] Stacey and Swift,[79] Jeanes, V and Miers,[44] and Haworth and Stacey.[36] Jeanes, Wilha Miers[45] passed dextran beer through a supercentrifuge to the bacterial cells before precipitating the dextran, but no is made of such a procedure by the other investigators.

Stacey and Yound[80] and Daker and Stacey[14] used 2 vol

for precipitation, whereas Tarr and Hibbert[illegible] used 5
.

ctional precipitation was utilized by Hehre and Neill,[illegible] in
a study of the polysaccharides produced by streptococci.
amples were diluted 1:10 with 10% sodium acetate and 1.2
volumes of 95% alcohol were added to separate portions of
mple. The sample treated with 1.2 volumes of alcohol was
an index of the dextran present, whereas the sample treated
volumes was considered to be a test for either dextran or

e only method described to date for the purification of
is that of reprecipitation.[illegible] Stacey and Swift[illegible] repre
d from water at different pH values until essentially ash-free
was obtained. Information on industrial methods was
d in 1953.[illegible]
lds of dextran reported in the literature vary from 10%[illegible]
[illegible] of the starting sucrose with most reports in the range of
5%.[illegible] The theoretical yield, assuming that only the
fraction of sucrose goes into the dextran molecule, would
oximately 47% of the sucrose.

The Structure of Dextran

mentioned previously, the true nature of dextran, as a
nd made up entirely of glucose units, was reported by
er,[illegible] in 1869. Information on how these molecules were
d, however, has appeared only in the last 20 years.
e complete methylation of dextran produced by one strain
onostoc mesenteroides was reported by Fowler, Buckland,
and Hibbert,[27] in 1937, to yield dimethyl, trimethyl, and
thyl methylglucosides in a ratio of 1:3:1. They conclude
ese results that dextran is a polymer of pentaglucopyranose
de units, one being attached as a side chain and the remain-
r most probably being connected in "linear chain union."
inkages are of the 1:6 type, while the other two may be
:6 or 1:4. It is possible that the side chains are composed
than one glucopyranose unit. These results were confirmed
Hawkins, and Hibbert,[illegible] in 1942, and again by Stacey and
in 1948.

Dextran produced by a strain of *Leuconastoc dextranicus* was reported by Fairhead, Hunter, and Hibbert,[24] in 1938, to show a very different result on methylation. No tetramethyl methylglucoside was formed. The product was principally the trimethyl derivative with about 10% of the dimethyl. This would indicate a very linear molecular arrangement with few side chains. Essentially the same results were reported by Peat, Schluchterer, and Stacey[70] the next year. Their results indicated a chain length o 200 to 550 glucose units. The same type of molecular arrangemen was found by Daker and Stacey[15] for a dextran produced by *Beta bacterium vermiforme* (Ward-Mayer). End-group determination indicated a chain length of 25 glucose units whereas the osmoti pressure method of molecular-weight determination indicated chain of about 500 units.

Uses of Dextran

Several ethers, esters, and mixed ether-esters of dextran we described by Stahly and Carlson,[82-99] in a series of patents, in 194 and 1941. Certain of these are described as suitable for use in th production of lacquers. The sulfuric acid ester of dextran w described by Grönwall, Ingelman, and Mosimann,[32] in 1945, having an anticoagulant potency comparable to that of hepari Other derivatives which have been described include the ac tate[112,113] and aryl carbamates.[117] The use of an acid ester as cation-exchange medium was described by McIntire and Schenck in 1948.

Dextran itself is a good stabilizer for sugar sirups,[57,111] i cream, and other confections and has been the basis for the ope tion of one company in the United States. It has also been su gested as a stabilizer for drilling muds for oil and gas wells.[4]

Interest in dextran was revived by the reports of Grönwall a Ingelman[29,30,31] on its use as a substitute for blood plasma in tra fusions. The experience of investigators with dextran in Swed and a number of reports of clinical investigations with this gene type have been well summarized by Ingelman,[47] in 1949. In t report, he described the product used as a partially hydrolyzed d tran with molecular weights in the range 20,000 to 200,000. drolysis was illustrated by an example in which an 8% solution raw dextran was heated to 90°C for 20 minutes in a solution

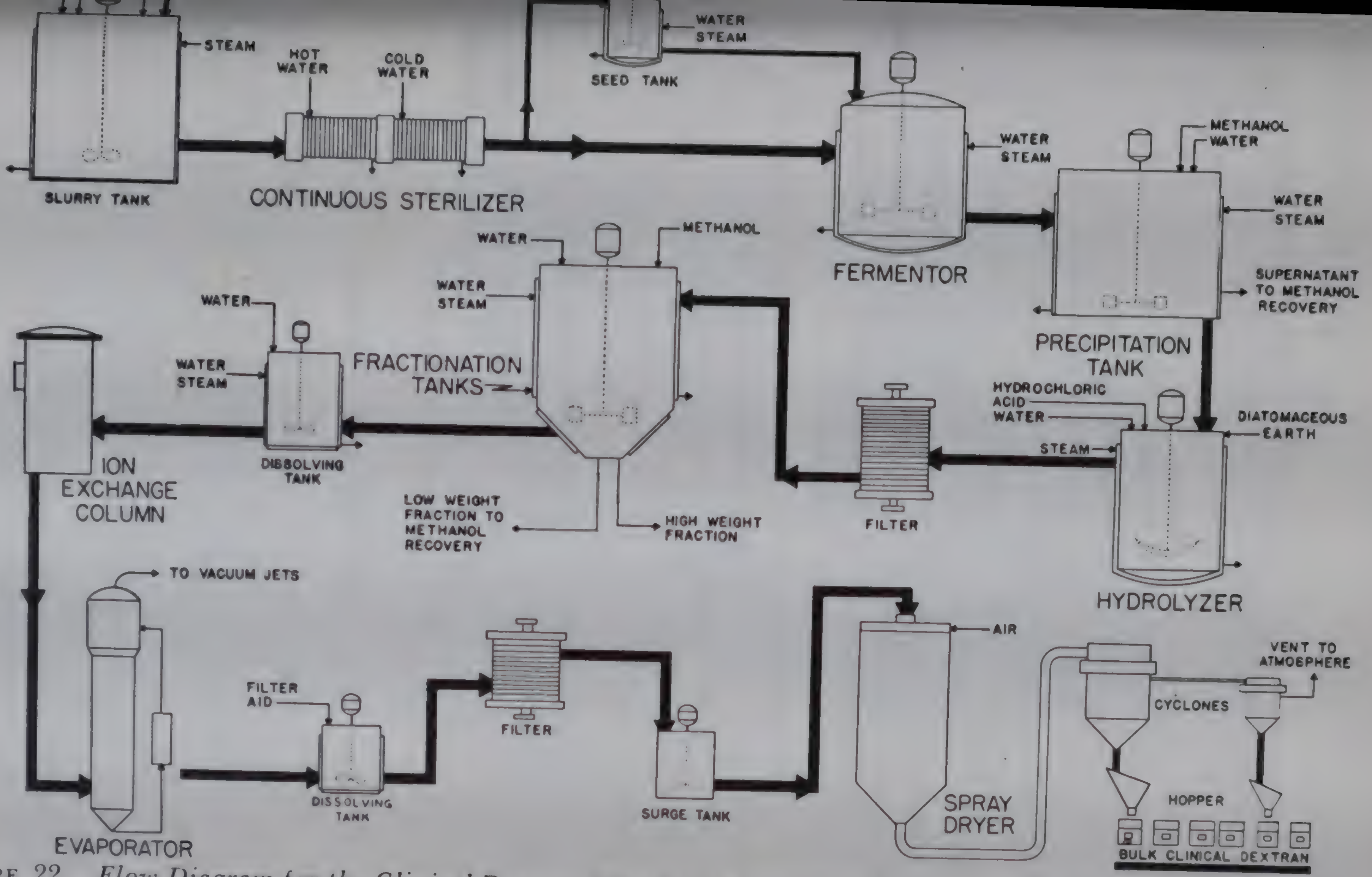

WATER
STEAM
SEED TANK
STEAM
HOT WATER
COLD WATER
SLURRY TANK
CONTINUOUS STERILIZER
WATER STEAM
FERMENTOR
METHANOL
WATER
WATER STEAM
SUPERNATANT TO METHANOL RECOVERY
PRECIPITATION TANK
WATER
METHANOL
WATER STEAM
FRACTIONATION TANKS
WATER
WATER STEAM
DISSOLVING TANK
ION EXCHANGE COLUMN
LOW WEIGHT FRACTION TO METHANOL RECOVERY
HIGH WEIGHT FRACTION
FILTER
HYDROCHLORIC ACID
WATER
STEAM
DIATOMACEOUS EARTH
HYDROLYZER
TO VACUUM JETS
EVAPORATOR
FILTER AID
DISSOLVING TANK
FILTER
SURGE TANK
AIR
SPRAY DRYER
CYCLONES
VENT TO ATMOSPHERE
HOPPER
BULK CLINICAL DEXTRAN

FIGURE 22. *Flow Diagram for the Clinical Dextran Process of Commercial Solvents Corporation* [Reprinted by permission from a paper by Bixler, Hines, McGhee, and Shurter, *Ind. Eng. Chem.*, **45**, 692 (1953)]

0.12 *N* hydrochoric acid. The hydrolysis was stopped by neutralization with sodium hydroxide and the dextran precipitated with alcohol and dried. Spray-drying is employed with success for commercial production of dextran.[8a] A neutral 6% (w/v) solution of this partially hydrolyzed dextran with a relative viscosity of 3.3 (compared to distilled water) may be sterilized by heating to 120°C for ½ hour. This type of a solution containing in addition 0.9% NaCl and passing certain specifications[8a] is used for injection.

Radioactive dextran employing C^{14} was prepared in 1952[78a] from sucrose which was photosynthesized by canna leaves which metabolized $C^{14}O_2$ supplied to them. Clinical dextran prepared in this manner was employed with success to follow the fate of injected material. Essentially all of the radioactivity was accounted for in expired CO_2 and in body excretions.

Recently,[1a,8a] it has been reported that four producers in the United States are supplying clinical dextran for use as a blood plasma extender. Production methods, equipment, and applicatio were described in some detail in 1953.[8a] The Armed Forces specif cations for clinical dextran were included in this report.[8a] A average molecular weight of 75,000 ± 25,000 as determined by ligh scattering was specified. The high molecular weight fraction (5 t 10%) should not exceed 200,000 and the low fraction (5 to 10% should not be less than 25,000. A flow diagram for the productio of clinical dextran at the plant of Commercial Solvents Corporatio in Terre Haute, Indiana, is shown in Figure 22.

THE SYNTHESIS OF *l*-EPHEDRINE

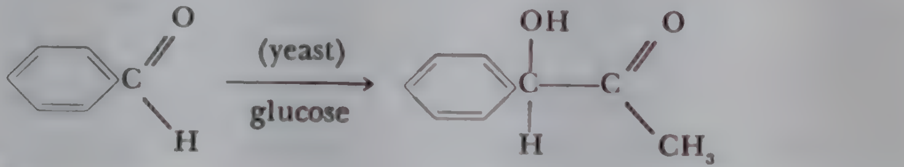
It has been commonly observed in drug studies that activi and utility of a drug may be dependent on its steric configuratio Great differences may be observed between the pharmacolog activities of *d*- and *l*-isomers of the same compound. For examp the *l*-isomer of ephedrine is about thirty times more active than t *d*-isomer in pressor properties. Chemical syntheses of many of t optically active drugs have been accomplished, but synthesis racemates has generally been involved and resolution is, to say t least, often troublesome and costly.

The alkaloid, *l*-ephedrine, has been successfully prepared the unique method of Hildebrandt and Klavehn,[44] described

1934, in which an asymmetric synthesis is accomplished fermentatively. An *l*-intermediate is prepared rather than the more common *dl*-intermediate.

Ephedrine was originally obtained from the Chinese plant, *ma huang*. The medicinal properties of the plant have been known to the Chinese for several thousand years. Ephedrine is a sympathomimetic amine which has been widely used for the relief of congestion in colds and in other disorders. The history and pharmacology of the drug have been discussed by Goodman and Gilman.[28]

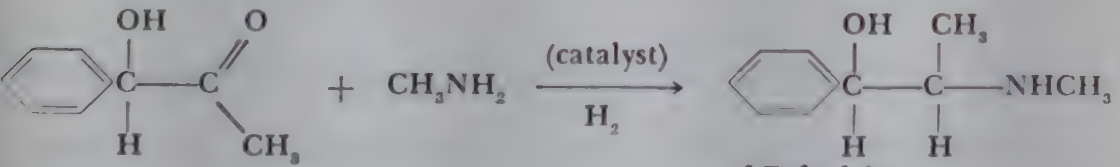
The Hildebrandt and Klavehn synthesis[44] makes use of the observations of Neuberg[65,66] who found, in 1921 and later, that top yeast in a glucose medium will fermentatively convert benzaldehyde to *l*-1-phenyl-1-hydroxy-2-propanone:



Benzaldehyde

l-1-Phenyl-1-hydroxy-2-propanone

The *l*-keto alcohol thus obtained is then reductively condensed with monomethylamine by chemical means to yield primarily *l*-ephedrine, along with pseudoephedrine, which may be separated from the ephedrine.

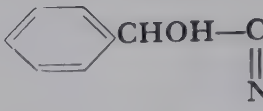


l-Ephedrine

The reduction is accomplished by means of aluminum amalgam or by catalytic hydrogenation using colloidal platinum.

The fermentative phase of the synthesis, according to Neuberg, would proceed, for example, by fermenting a mixture of 125 g of glucose sirup and 100 g of yeast in 2.5 l of solution for ½ hour. Following this, 10 g of benzaldehyde is added in increments with stirring. At the end of 3 days, the yeast is removed and, by ether extraction and subsequent isolation procedures, about 2.5 g of *l*-1-phenyl-1-hydroxy-2-propanone is obtained, along with significant amounts of benzyl alcohol and benzoic acid.

Hildebrandt and Klavehn[44] cite severa
for conversion of the ketoalcohol to ephed
to a solution of 100 g of *l*-1-phenyl-1-hyd
ml of ether, 75 g of a 33% solution of mo
The mixture is agitated for about 30 min
an exothermic condensation occurs yieldin



The mixture is then catalytically hy
of 1% colloidal platinum. The *l*-ephed
crystalline hydrochloride after filtration
drochloric acid. The yield of final produ
example; in a related example employi
material, about 110 g of ephedrine hydroc
300 g of crude ketoalcohol oil. The fac
metric undoubtedly affects the proportio
reduction.

Studies on the mechanism of bio
hydroxy-2-propanone (phenylacetylcarbi
Smith and Hendlin[78c] in considerable det

It is to be expected that modifications
are employed in actual practice.

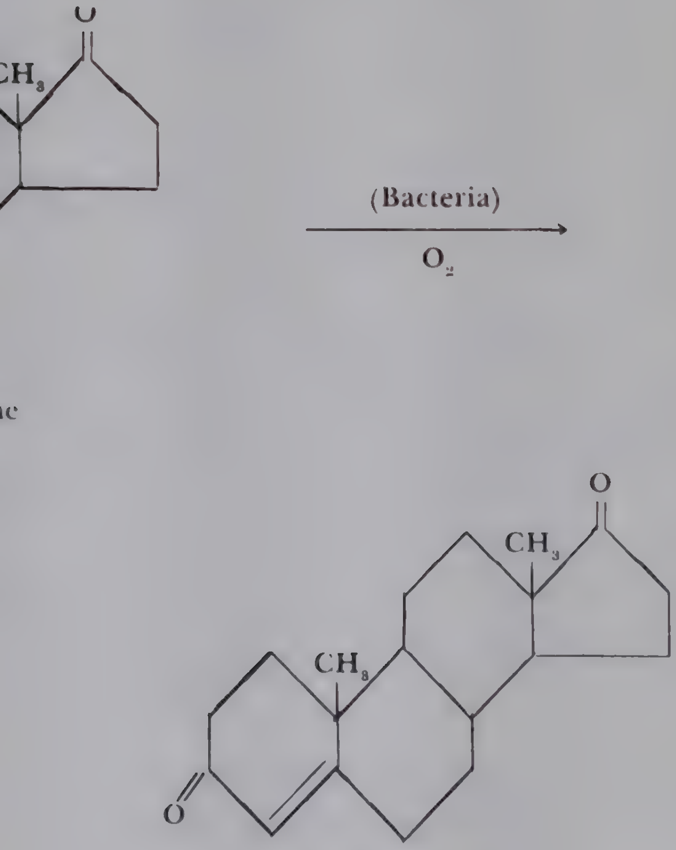
THE ACTION OF MICROORGAN

Steroid reactions have been accom
generally by purely chemical means. Th
steroids, such as bile acids, sex hormones,
tial to biological processes indicates,
reactions of steroids occur. Reactions
organisms might be expected and some
observed. Mildness of reaction conditi
oxidation by-products have been note

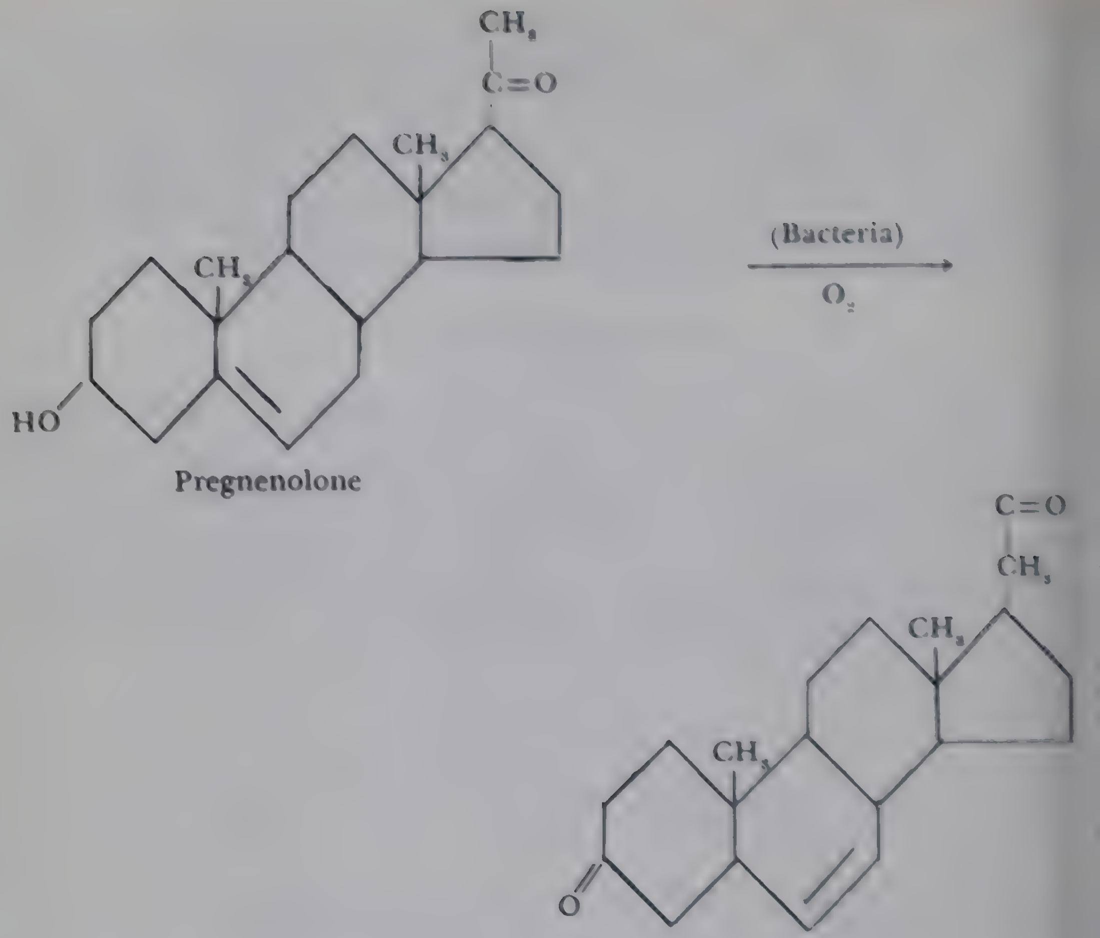
xidations have been described in which a
ituted for hydrogen directly on the ring
subject was reviewed fairly extensively
r;[26a] direct introduction of oxygen on a
eported until about 1947.[50a]

xidative Reactions

escribed the conversion of dehydroandro-
ie by bacteriological oxidative means. The
probably *Flavobacterium helvolum*[59] or
;anism. *Acetobacter pastorianum* has also
e this reaction which was described further,
h, and Teschen.[60]



Androstenedione

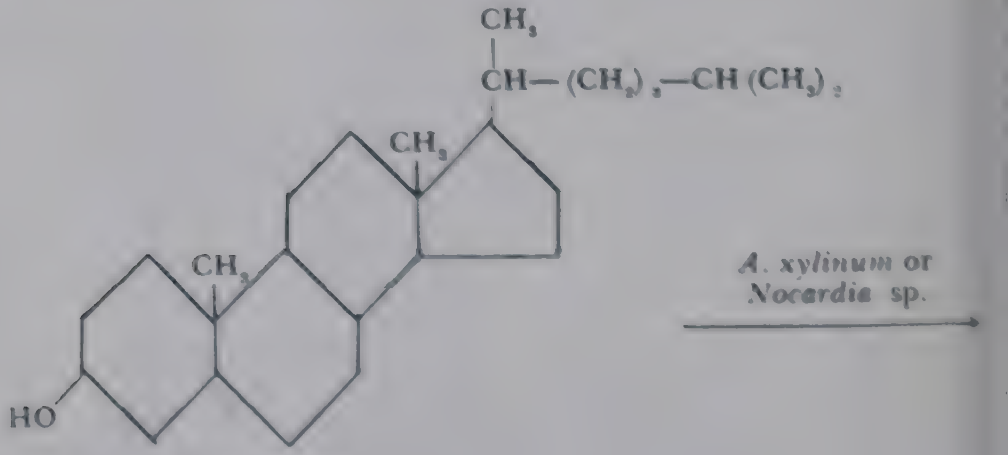


CH₃
C=O
CH₃
CH₃
HO
(Bacteria)
O₂
C=O
CH₃
CH₃
CH₃
O

Pregnenolone

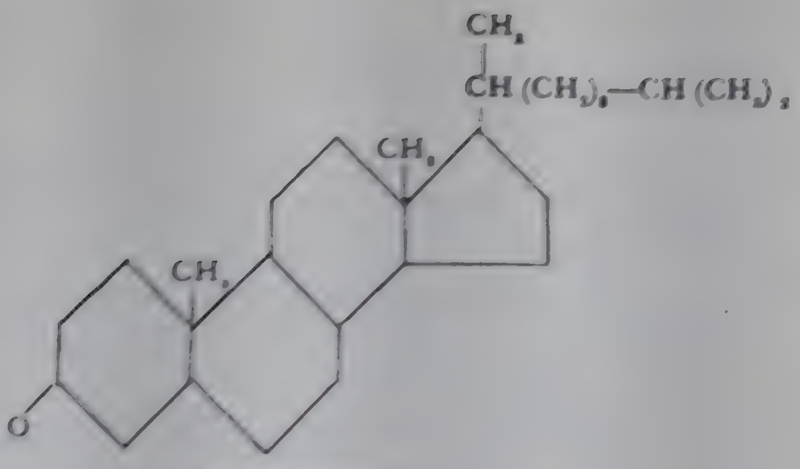
Progesterone

In addition, Mamoli[58] attempted to oxidize cholesterol means of *Fl. helvolum*, but was unsuccessful. Subsequently it w found that both *Acetobacter xylinum*[50] and species of *Nocardia* would oxidize cholesterol to △4-cholestenone:



CH₃
CH—(CH₂)₃—CH(CH₃)₂
CH₃
CH₃
HO
A. xylinum or
Nocardia sp.

Cholesterol

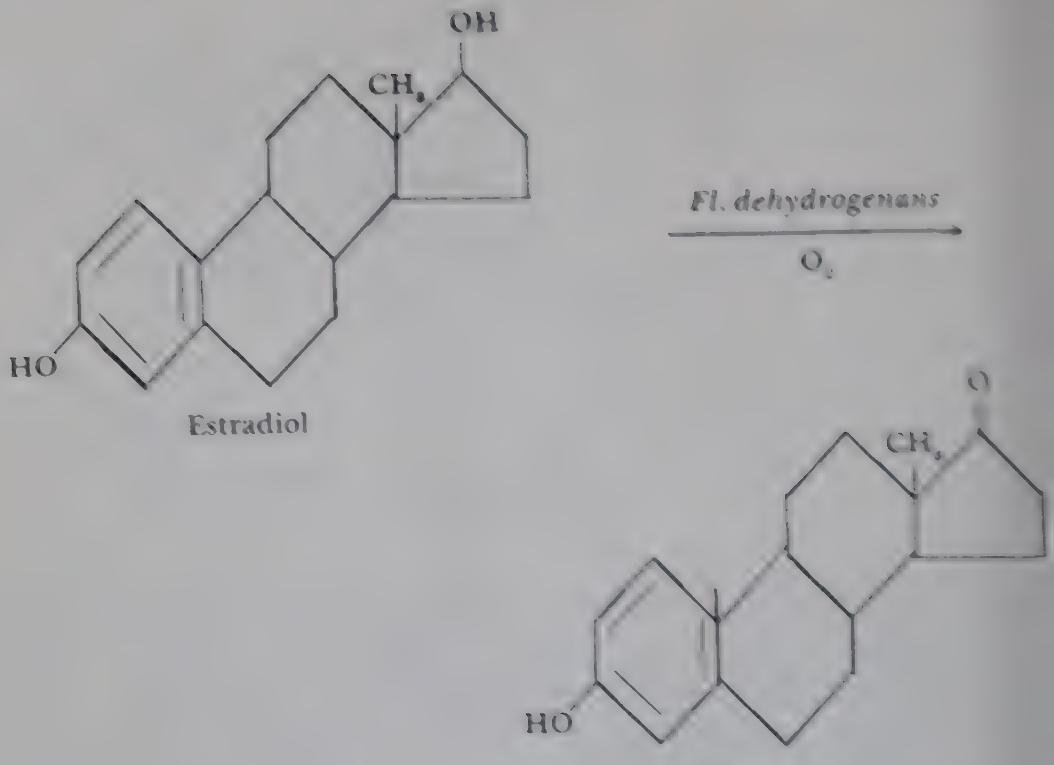


Cholestenone

For this reaction, Turfitt[103] employed a medium of inorganic lts, with the sterol as the only organic carbon source. Flasks oculated with *Nocardia* were incubated statically for 1 month 25°C prior to recovery of the product. Turfitt[104,105,106] has also ported other related investigations. In 1949, Schatz Savard, and intner[75] described studies with a number of *Nocardia* and other ngi, along with several bacteria. Both static and shake flasks were nployed; more rapid metabolism was found in the shake flasks. he presence of calcium carbonate in the media as a buffer was dvantageous to fermentative attack. Steroid conversions were not iscussed.

Hughes and Schmidt[46] showed, in 1942, that a strain of *lcaligenes faecalis* in nutrient medium, occasionally agitated, ould oxidize desoxycholic, hyodesoxycholic, and lithocholic acids nd also dehydroisoandrosterone to corresponding ketoderivatives. rior studies of a similar nature[77,78] showed that *A. faecalis* would so oxidize cholic acid to ketocholanic acids. Hughes and Schmidt[46] so attempted the oxidations of estradiol and estriol under similar nditions, but were unsuccessful.

In 1941, Ercoli[21] observed that estradiol could be converted to trone by the use of a heavy culture of *Flavobacterium dehydro-nans (Micrococcus dehydrogenans)*. Other steroid reactions were so observed to be brought about by this organism.[19,20] Similarly, 1944, Zimmerman and May[119] found that pseudodiphtheria acilli were capable of converting estradiol to estrone and an-rostenediol to testosterone.



Estradiol

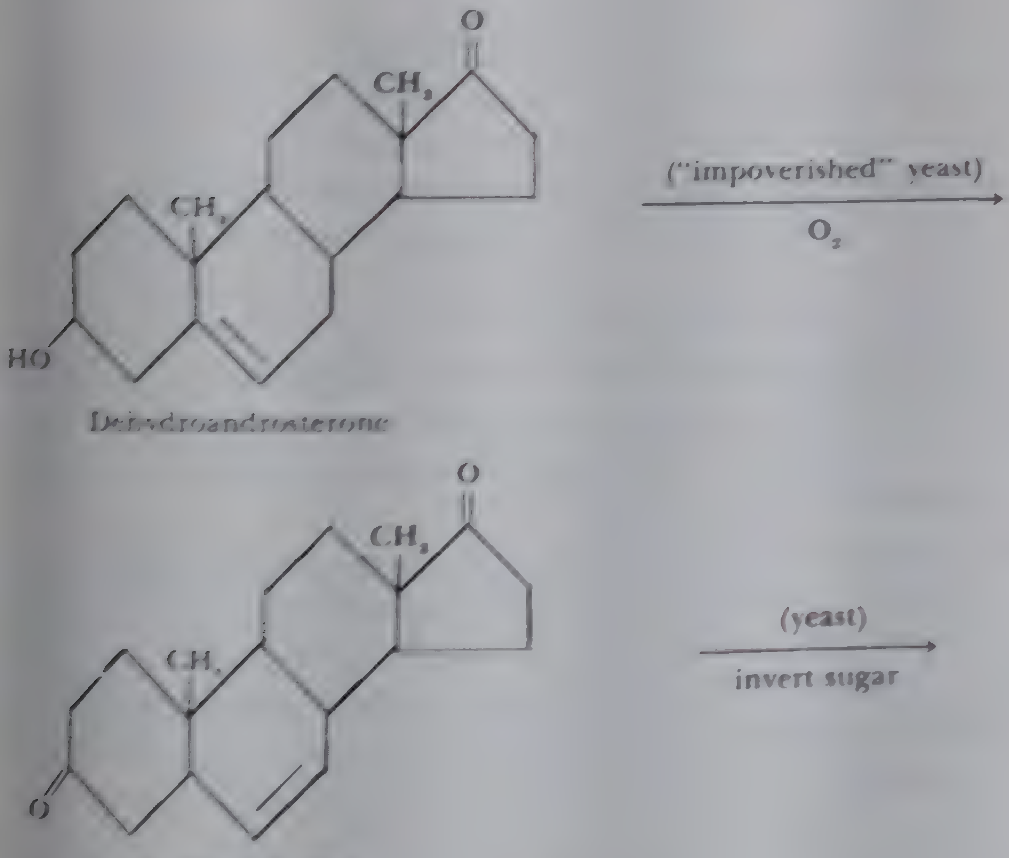
Estrone

Similar findings were reported, in 1942, by Arnaudi[2] showed that *Fl. dehydrogenans* would convert pregnenolone progesterone, dehydroandrosterone to androstenedione, and es diol to estrone, along with other reactions. In 1944, Ercoli Molina[22,23] reported a new organism, *Fl. androstenedionicum*, wh they found would convert androstenediol to androstenedi Another organism, *Fl. carbonilicum*, behaved somewhat s larly;[64] it was found to oxidize androstenediol to a mixture testosterone and androstenedione. The subject was studied furt in 1946, by Arnaudi.[3]

As a specific example of microbiological steroid oxidat Mamoli[59] has claimed, in a 1944 patent, a method for conver of 21-acetoxypregnenol-3-one-20 to desoxycorticosterone. In procedure, the medium employed was composed of 60 ml st yeast water buffered with 10 ml each of 0.2 *M* Na_2HPO_4 and 0 KH_2PO_4. To this medium 200 mg of pulverized 21-acetoxy nenol-3-one-20 was added and the mixture was sterilized for 1 h The cooled, sterile medium was then inoculated with a cultu *Flavobacterium helvolum* ("*Bacterium Coryne*"), after which tation was employed for 6 days under an oxygen atmosphere the end of this period, the product was filtered, the residue lved in acetone, and the bacterial residue was removed from cetone extract by filtration. On removal of the acetone by oration, the residue was recrystallized with care from "diluted one." The first crystal crop of 54 mg was composed of unted 21-acetoxypregnenol-3-one-20. The mother liquor was evaped and the residue was taken up in acetone. After diluting this one solution with ether, a 60 mg crop of crystals was obtained ch was identified as desoxycorticosterone, M.P. 139° to 140°C. er related dehydrogenation reactions were also described.

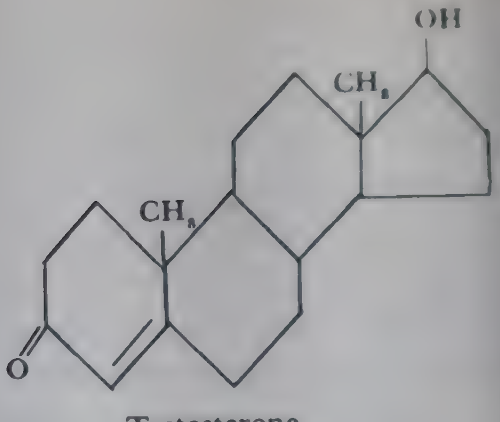
Additional reactions were set forth in a patent of Koester, moli, and Vercellone.[50] Microorganisms employed by these inigators included both yeast and *Acetobacter*. In one example d, *Acetobacter pastorianum* in surface culture converted anstenediol to testosterone. Similarly, androstenediol monopronate-17 was oxidized to testosterone propionate.

An example was also cited[50] in which a Milan yeast was emyed for both oxidation and reduction to convert dehydrodrosterone to testosterone:



Dehydroandrosterone

Androstenedione

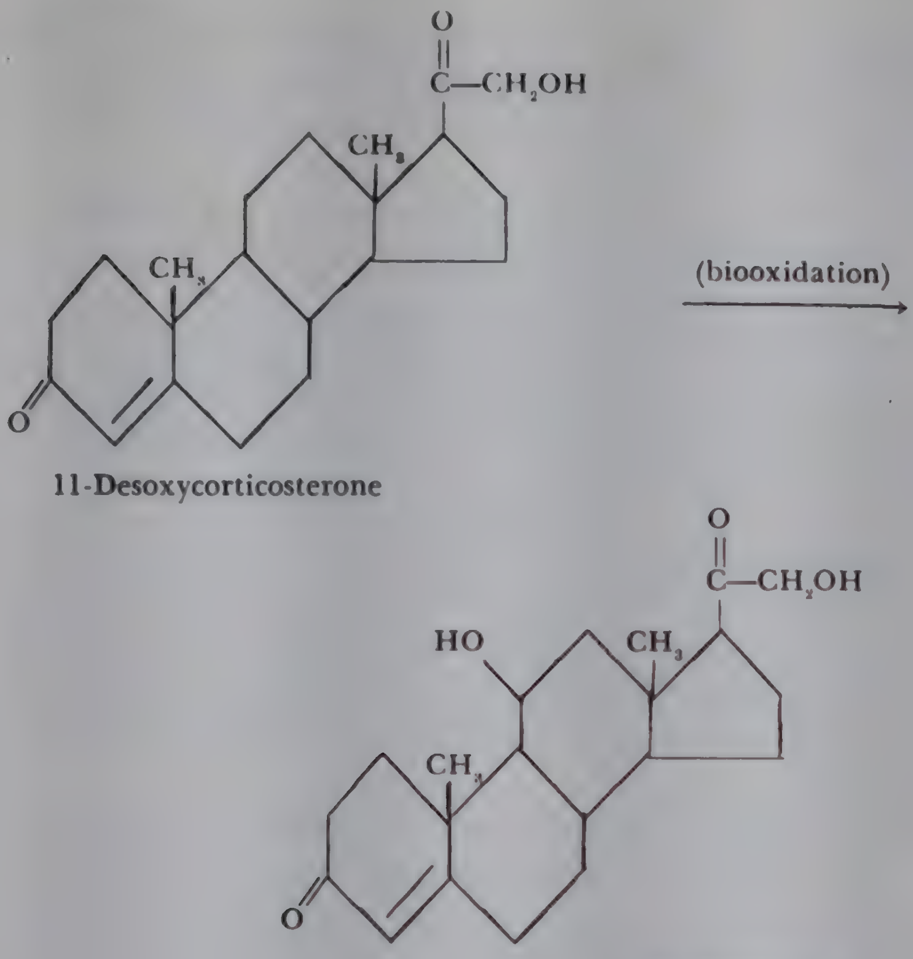


Testosterone

Experimentally, 8 g of yeast in 50 ml of phosphate buffer wa shaken for 20 hours at 32°C in an oxygen atmosphere. This pr cedure produced an "impoverished" yeast. To the mixture, 200 m of dehydroandrosterone, suspended in 30 ml of water, was adde The resulting mixture was then shaken for an additional 48 hour under oxygen. At this point, 25 g of invert sugar, dissolved in 15 ml of water, was added and the mixture was allowed to stand an ferment for 3 days at room temperature. From this mixture, 12 mg of testosterone was recovered.

In the last few years, there have been considerable activit and success in conducting biological oxidations of steroids for in troducing oxygen at carbons of the steroid nucleus. In 1947 an 1949, Krámli and Horváth[50a] reported that 7-hydroxycholester was derived from cholesterol by microbiological oxidation wit *Proactinomyces roseus* or by *Azotobacter* species. Perlman, Titu and Fried[71a] have recently reported that an unidentified actin mycete was found which would convert progesterone in simple n trient media to 16-α-hydroxyprogesterone in good yield. In add tion, small amounts of pregnanol-16-α-dione-3,20 and a dihydrox progesterone were produced.

In 1949 and later, Hechter, Jacobson, Jeanloz, Marshall, Pinc and Schenker[37,38] demonstrated that oxygen could be introduced carbon 11 by perfusion of steroid-containing fluids through adren glands. This was, of course, a very highly selective oxidation a was quite remarkable from the organic chemist's point of view, sin the 11-position is considered as highly inaccessible. Introducti of oxygen at carbon 11 in this manner was an important and uniq step in the field of corticosteroid syntheses. An example of such oxidation is as follows:



11-Desoxycorticosterone

Corticosterone

Other related investigations have been described, and further studies of adrenal biooxidations of steroids have been reported.[36a,38a,117a]

In 1952, Peterson and Murray,[71b,71c] working in the research laboratories of the Upjohn Company, Kalamazoo, Michigan, reported that they had been able to oxygenate steroids at carbon 11 by means of a fungal biooxidation, employing certain *Mucorales*. As an example, *Rhizopus arrhizus* was utilized effectively for the oxidation of progesterone to 11-α-hydroxyprogesterone in a medium containing lactalbumin digest, corn steep and glucose. The oxidation required 24 to 48 hours and resulted in yields of 10% and higher. Along with the monohydroxy compound, dihydroxy steroids were also isolated. Similar biooxidations at carbon 11 occurred with other steroids, including androstenedione, 11-desoxycorticosterone and 11-desoxy-17-hydroxycorticosterone. The 1952 patent of Murray and Peterson[71c] cites many examples of the general procedure, including variations in substrate and culture. Paper

chromatographic analytical methods were described. These we
based on the technique of Burton, Zaffaroni, and Keutmann.
Additional paper chromatographic methods have been described
Shull, Sardinas, and Nubel.[78b]

A series of papers was presented subsequently in 1952[71g] and
1953[18a,18b,63a,63b,71c,71h] which detailed a number of specific stero
transformations of the general type noted in the 1952 patent of Mu
ray and Peterson.[71c] In addition to the microbiological oxidati
transformations of steroids, many chemical transformations are al
described in these papers. The purely chemical reactions of steroi
are outside the scope of this book (see also Peterson[71b]).

In addition to *Rhizopus arrhizus* Fischer (ATCC 11145), whi
was a specific strain employed, *Rhizopus nigricans* Ehrb (ATC
62276) has been used for oxygenation of steroids at carbon 11. F
example, this second fungus was reported[63a] to convert 4,16-pre
nadien-3,20-dione to 11-∝-hydroxy-17∝-progesterone under oxid
tive conditions.

Oxidation at C-11 is not always obtained with these *Rhizop*
species. For example, it was reported[18a] that, although *Rhizop*
nigricans converted 11-desoxycorticosterone (or its acetate) to
epicorticosterone, *Rhizopus arrhizus* converted this substrate to 6
hydroxy-11-desoxycorticosterone. Introduction of oxygen into t
ring by the second culture was accomplished at position 6 rath
than at position 11, in contrast to *Rhizopus nigricans* which
tacked position 11.

In 1952, Colingsworth, Brunner, and Haines[13a] reported th
Streptomyces fradiae No. 3535 (Waksman) could also oxid
steroids at carbon 11. As an example, 5 g of 11-desoxy-17-hydro
corticosterone (Reichstein's compound S) was dispersed in 1
medium containing glucose, soybean meal, and distillers' solubl
This was incubated in rotary shake flasks with *S. fradiae* for 7 ho
at 24°C. From this mixture 4.86 g of a neutral hormone concentr
was obtained which contained, according to bioassay, 140 mg of
hydroxycorticosterone (Kendall's compound F). Purification w
accomplished by partition chromatography. A trace of 11-dehyd
17-hydroxycorticosterone was also detected.

In addition, according to Fried and associates,[27a] in 1952, cert
members of the genus *Aspergillus* can also oxygenate at carbon
For example, *Aspergillus niger* (Wis. 72-2) was reported to att

progesterone, Reichstein's compound S, desoxycorticosterone, and 17α-hydroxyprogesterone in this manner. Progesterone, as an example, gave a mixture of 11α-hydroxyprogesterone in 35% yield and 6ξ, 11-α-dihydroxyprogesterone in 20% yield.

A patent was issued in 1953 to Shull, Kita, and Davisson* of Chas. Pfizer & Co., Inc., pertaining to the hydroxylation of steroids at the 11 position, using the genus *Curvularia,* or an enzyme preparation from these organisms. For example, *Curvularia lunata* (NRRL-2380) was grown aerobically for 22 hours in a medium consisting of salts, sucrose, and malt extract. The mycelium from 2 l of such a fermentation, for example, is separated by filtration. It is then washed and suspended in an equal volume of distilled water. Such a preparation was found to be capable of converting 0.5 g of 17-hydroxy-11-desoxycorticosterone (added in 20 ml ethanol solution) after 16 hours of aeration to 17-hydroxycorticosterone in 35% yield. Purification involved extraction with chloroform and chromatography on silica gel. In a similar manner, *C. pallescens* (NRRL-2381) brought about the conversion of desoxycorticosterone to corticosterone. A 28% yield was obtained.

Some degradations of steroids were also reported recently to be brought about by certain other fungi;[71d] side chains were removed. Among the fungi capable of doing this are *Gliocladium catenulatum, Penicillium lilacinum* Thom, *Aspergillus flavus,* and *Penicillium adametzi.* For example, progesterone may be degraded to androstenedione and other oxygenated steroids by *Gliocladium catenulatum.*

In view of the importance of cortisone and other adrenocortical steroids in medicine, some of these discoveries may be considered as major accomplishments in the advancement of biological science.

Reductive Reactions

The microbiological reductive reactions of steroids have generally involved the reduction of ketone groups to hydroxyl groups.

In 1937, Mamoli and Vercellone[61] described several microbiological reductions of ketosteroids for which yeast was used as the reducing organism. These investigators demonstrated, for ex-

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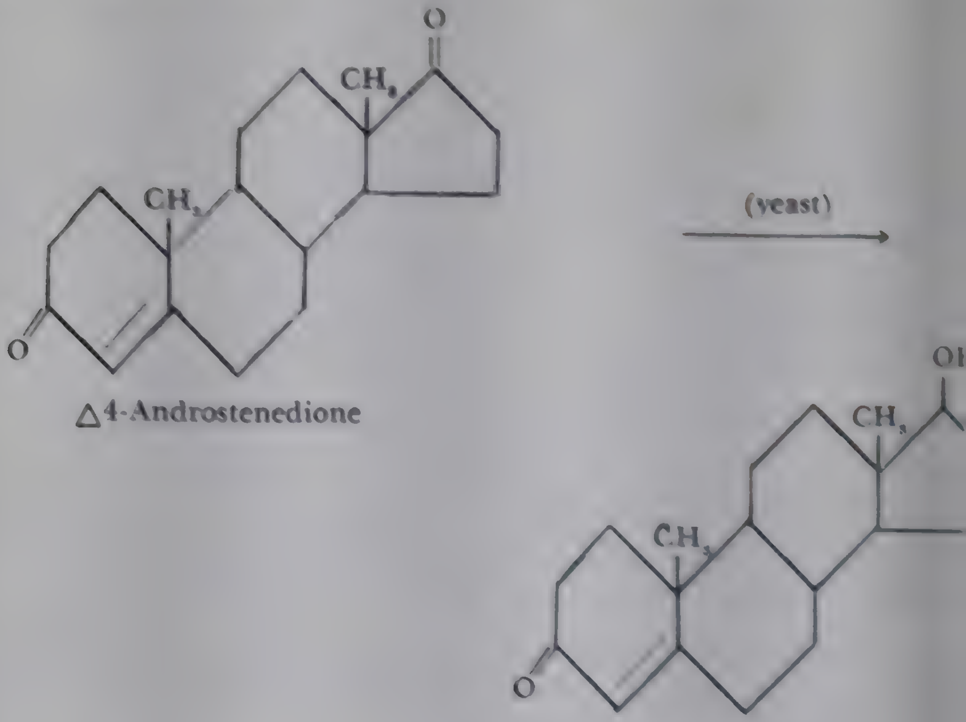
* Shull, G. M., D. A. Kita, and J. W. Davisson, U. S. Patent 2,658,023 (1953). [*C.A.* **48**, 2332 (1954)].

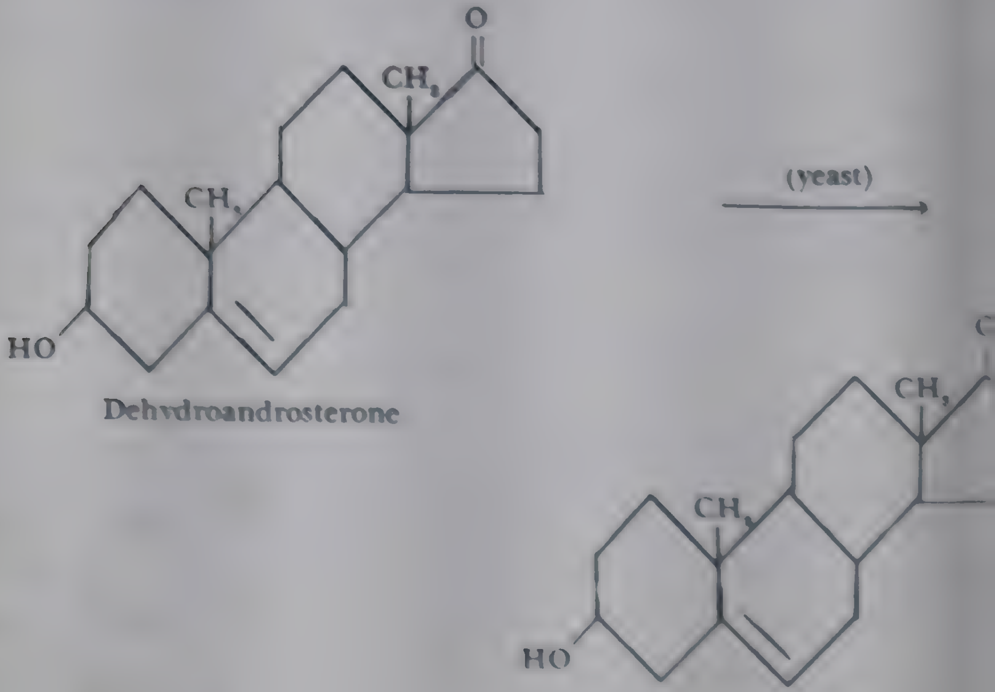
ample, that Δ4-androstenedione could be reduced by the
of yeast to testosterone:


O
CH$_3$
CH$_3$
O
(yeast)
OH
CH$_3$
CH$_3$
O

Δ4-Androstenedione

Testosterone

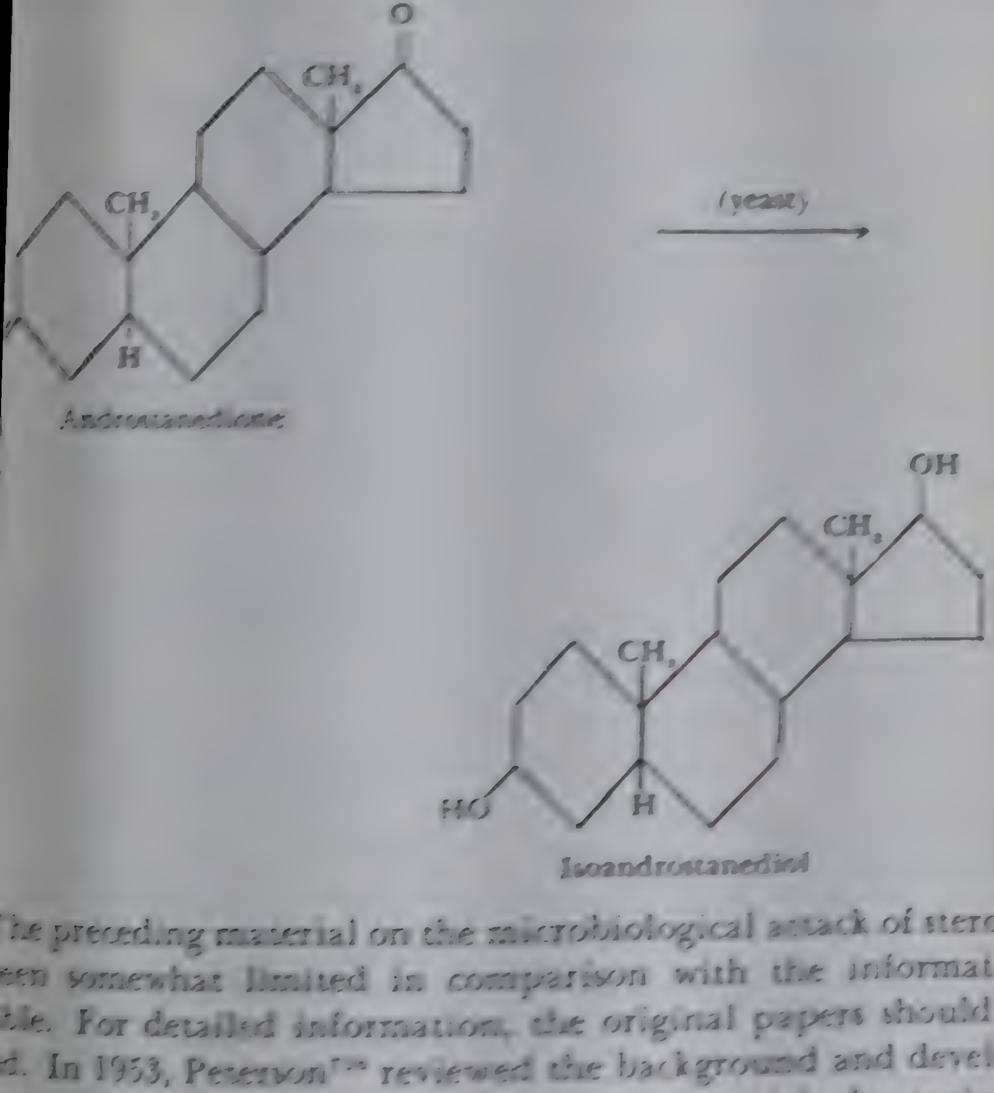
By the same general procedure. i.e., reduction by yeast a
tion 17, these investigators[62] also were able to reduce de
androsterone to androstenediol:


O
CH$_3$
CH$_3$
HO
(yeast)
CH$_3$
CH$_3$
HO

Dehydroandrosterone

Androstenediol

rcellone and Mamoli[illegible] were able to obtain reduction of the group as well as at position 17. This is seen from the fact could reduce androstanedione to isoandrostanediol, again se of yeast. There was no ring unsaturation in this example:



Androstanedione

Isoandrostanediol

he preceding material on the microbiological attack of steroids een somewhat limited in comparison with the information ble. For detailed information, the original papers should be d. In 1953, Peterson[illegible] reviewed the background and development leading to the preparation of cortisone and hydrocortisone gh microbiological approaches. Considerable additional ref- s and information may be found in this report.

t the present time, it would be difficult or impossible to de- e the extent of commercial utility of the various specific d reactions noted and to indicate their relative values. Re- s resulting in improved means for the economic preparation isone and hydrocortisone would undoubtedly be potentially ually of economic value. The results of investigations such as should have considerable additional value in suggesting that other organic chemical reactions which are difficult to ac-

complish might be brought about by the proper application
microorganism or enzyme reactions.

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CHAPTER 11

LACTIC ACID FERMENTATION OF CUCUMBERS, SAUERKRAUT AND OLIVES

Reese H. Vaughn

INTRODUCTION

Preservation of foods by lactic acid fermentation (pickling) has been one of the important methods of food preservation for centuries. Until the development of canning and freezing processes during the past 150 years, pickling and drying constituted the major methods for preserving foods. However, with the development of canning and freezing techniques, food habits have changed, particularly in the United States. Now the pickled foods, cucumbers and olives especially, are used as food adjuncts which function as appetizers.

The most important pickled products still produced in whole or in part by lactic acid fermentation include cucumbers (pickles), cabbage (sauerkraut), and olives. That these pickled foods still constitute an important segment of the food industry of the United States is shown by production figures.

Cucumbers probably constitute the most important single crop produced for pickling. Michigan and Wisconsin lead in the production of cucumbers in the eastern United States, while California leads among the western states. In 1947, Michigan, Wisconsin, and

California harvested 35,600, 18,900, and 2,020 acres, with yields pe acre of 1.5, 2.4, and 8.3 tons, respectively. Data showing the numbe of tons of salt stock and dill pickles produced in the United State are found in Table 38. It should be remembered in addition tha the cucumber pickle is a popular and extensively home-prepare

TABLE 38. COMMERCIAL PRODUCTION OF CUCUMBER PICKLES[a] IN THE UNITED STATES

Year	Salt stock tons	Dills tons	Total tons	Ratio of salt stock to dills
1930	138,048	35,592	173,640	3.87
1931	110,208	23,184	133,392	4.75
1932	28,080	11,160	39,240	2.52
1933	61,440	16,680	78,120	3.68
1934	79,896	12,912	92,808	6.18
1935	93,648	14,616	108,264	6.41
1936	117,024	17,784	134,808	6.58
1937	138,144	25,944	164,088	5.32
1938	119,808	16,680	136,488	7.18
1939	70,752	13,968	84,720	5.06
1940	116,040	18,888	134,928	6.14
1941	151,776	18,912	170,688	8.03
1942	155,736	20,520	176,256	7.59

[a] Calculated from data in *Western Canner and Packer,* **35**, No. 5, 221 (194 on the basis of 48 lb per bu of cucumbers.

TABLE 39. COMMERCIAL PRODUCTION OF SAUERKRAUT IN THE UNITED STATES

Year	Total canned, cases, all sizes		Total bulk sauer kraut in barrels (45 gal per barre (thousands)
	Sauerkraut (thousands)	Sauerkraut juice (thousands)	
1925	2,395	—	—
1927	3,101	—	—
1929	4,224	—	—
1931	3,645	69	—
1933	3,345	—	—
1935	4,405	120	—
1937	4,583	234	—
1939	4,458	135	—
1940	6,480	—	—
1941	6,202	—	360
1942	3,062	—	460
1943	3,012	—	160
1944	2,537	—	160
1945	6,076	—	360
1946	5,904	—	460
1947	1,650	—	120

[a] Tin and glass containers.
Source: *Western Canner and Packer,* **40**, No. 5, 135 (1948).

TABLE 40. CALIFORNIA OLIVE PRODUCTION COMPARED WITH IMPORTS FROM SPAIN

	Total California crop	California canned ripe olives		California green olives				Total imported green olives		
				Sicilian type		Spanish type				
Season[a]	tons	tons	gallons[b] (thousands)	tons	gallons[b] (thousands)	tons	gallons[b] (thousands)	year[c]	tons[d]	gallons[b] (thousands)
1940-41	69,000	16,200	5,890	4,200	1,527	5,100	1,854	1941	15,529	5,647
1941-42	55,000	16,700	6,072	1,600	582	4,500	1,636	1942	14,039	5,105
1942-43	57,000	11,100	4,036	1,900	691	5,500	2,000	1943	23,914	8,696
1943-44	57,000	15,500	5,636	1,300	473	5,200	1,891	1944	26,958	9,803
1944-45	43,000	13,800	5,018	500	182	3,600	1,309	1945	25,251	9,182
1945-46	30,000	13,500	4,909	500	182	3,000	1,091	1946	29,384	10,685
1946-47	48,000	27,500	9,999	500	182	3,300	1,200	1947	17,966	6,533
1947-48	40,000	13,700	4,982	1,100	400	2,600	945	1948	30,583	11,121
1948-49	58,000	13,500	4,909	1,200	436	5,700	2,073	1949	17,498	6,363
1949-50	35,000	20,400	7,418	800	291	2,600	945	1950	34,326	12,482
1950-51	42,000	24,900	9,055	1,200	436	2,600	945	1951	28,174	10,245
1951-52	64,000	33,300	12,109	1,300	473	3,300	1,200	1952	37,164	13,514
1952-53	57,000	24,400	8,873	900	327	4,500	1,636	—	—	—

[a] California olive season begins in September.

[b] A gallon is approximately equal to 5.5 lb of olives.

[c] Import data available on calendar year basis.

[d] Includes all imported green olives, whole, pitted, pitted and stuffed.

Sources: California Olive Association; United States Department of Commerce, Foreign Commerce and Navigation of the United States.

food, particularly in the rural areas throughout the United States.

Sauerkraut (fermented cabbage) is the only important pickled vegetable product other than cucumbers fermented in any significant quantity in the United States. Its commercial production is localized largely in New York, Wisconsin, Ohio, Indiana, and Michigan, other states bordering on the Great Lakes, and in Colorado and Washington. Sauerkraut is a very popular food among the people of northern European extraction, especially among the Germans, as its name suggests. Information concerning the amount of sauerkraut produced commercially is found in Table 39. Sauerkraut is esteemed for its taste as well as its economy. It is produced in quantity in the home.

California produces almost all of the olives grown in the United States. There are some 25,000 bearing acres which normally produce crops ranging from about 30,000 to nearly 70,000 tons per season. Although olives may be grown throughout the state, most of them are grown in the two large interior valleys, the San Joaquin Valley and the Sacramento Valley. Arizona is the only other state which produces a commercial quantity of olives. Data concerning recent California olive production and the chief type of pickled olives produced are shown in Table 40. These data do not include fresh shipments; the quantities used on the farms in California

TABLE 41. VALUE OF FERMENTED PRODUCTS PROCESSED IN THE UNITED STATES

Product	Dollar value	
	1939	1947
Pickles (all kinds)	44,876,000	105,647,000
Sauerkraut		
Canned	6,439,075	8,705,000
Bulk	2,101,373	1,642,000
Juice	180,398	(not available)
Total value	8,720,846	10,347,000
Olives		
Ripe[a]	3,116,395	15,950,000
Bottled[b]	6,127,410	16,735,000
Total value	9,243,805	32,685,000
VALUE OF ALL PRODUCTS	62,840,651	148,679,000

[a] Produced in California.

[b] Produced in California and in Spain—final processing in the United States green olives.

Sources: United States Bureau of Census, 16th Census of the United States 1940. Manufacturers, 1939, Vol. 2, part 1, pages 114, 125-128, U. S. Government Printing Office, Washington, D. C., 1942.

United States Bureau of Census, Census of Manufacturers: 1947, Vol. 2, pages 97, 98, U. S. Government Printing Office, Washington, D. C., 1949.

those utilized to produce chopped ripe olives; oil; Greek-type olives and other processes; or the small commercial crops of Arizona.

The total value of pickles, sauerkraut, and olives processed in the United States in 1939 and 1947 is shown in Table 41. The chemical, physical, and microbiological aspects of the production and processing of each of these pickled foods will be discussed separately.

THE PICKLING OF CUCUMBERS

The cucumber (*Cucumis sativus*) is one of the oldest vegetables cultivated by man. It is supposed to have had its origin in Asia, perhaps in India, at least 3,000 years ago. It is popular both as a fresh and as a pickled vegetable and is grown widely in temperate climates although it was of semitropical origin. However, care must be taken to protect it against frost and drought and to control its microbial parasites and insect pests.

Pickling cucumbers must be grown from varieties known to have regular form, firm texture, and good pickling qualities. The common pickling varieties recommended by various authorities include the Chicago pickling, Boston pickling, Jersey pickling, Snow's perfection, National pickling, Heinz pickling, Fordhook pickling, Packer, Davis blend and various strains.[53,82]

Pickling cucumbers are harvested while still immature. Fully mature (ripe) cucumbers are undesirable for pickling, because they become too large, change color and shape, and are too soft for most commercial uses. Care must be taken in picking and transporting the cucumbers to avoid undue bruising and crushing. After picking, it is mandatory to deliver the cucumbers to the salting station or factory as soon as possible to prevent deterioration. Sorting to remove defective and distorted cucumbers (wilt, rot, nubbins, crooks, etc.) is done before salting or brining. In order to minimize spoilage during fermentation, it is very important to remove all unsound and decomposed cucumbers. Prior to fermentation, the cucumbers are generally graded for size with mechanical graders or by hand sorting. Final size grading is performed at the completion of the fermentation process. Some factories grade into four or more sizes; others remove only the large cucumbers for dills and ferment the rest field run.

There are two main types of pickled cucumbers made: salt-stock pickles from which the majority of the well-known sour, sweet, mustard, processed or manufactured dills, mixed pickles, etc., are made and fermented dill pickles.

Preparation of Salt-Stock Pickles

The cucumbers must undergo a preliminary fermentation and curing in brine before they are suitable for processing and finishing into sweet, sour, spiced, and other kinds of pickles. The fermentation takes place in circular tanks or vats of 8 to 14 or more fee diameter, and of 6 to 8 or more feet height. These fermentation tanks are usually made of cypress, redwood, fir, or pine, and sometimes reinforced concrete, and have capacities for 100 to 1,600 bu (2.4 to 38.4 tons) of cucumbers.

The salt-stock tanks may be located at the factory or near the center of the production area. If located apart from the processing and finishing factory, the collection of tanks is commonly called a salting station. Many of the salting stations are frame building which house, or partially house, the salting tanks and receiving platforms. Others are little more than a collection of tanks with a suitable receiving platform.

There are two general methods for making salt-stock pickles dry salting and brining. The dry-salting technique is not use extensively because of the tendency to yield soft, flabby, shrivele pickles that do not fill out properly when processed.

DRY-SALTING PROCEDURE

Dry salting is accomplished by first adding 40° Salometer sal brine to the tank to a depth of 12 in. to prevent bruising, breaking or crushing of the fresh cucumbers when they are dumped into th tank. The salt hydrometer (salometer, salimeter, or salinometer) i calibrated so that the Salometer degrees equal the percentage satura tion of salt (NaCl) at 60°F. A saturated solution of pure sodiun chloride (100° Salometer) contains 26.359 g salt at 60°F. Dr salt is added at the rate of about 50 lb for every 1,000 lb of smal cucumbers, and 65 lb for every 1,000 lb of large cucumbers. Whe full, the tank is covered with a weighted, circular, slatted, woode head until there is room for about 6 in. of brine above the cove The weighted head is then secured by heavy cross timbers held a the ends with clamps. If the brine does not cover the pickles o wooden cover when the tank is closed, 40° Salometer brine is adde to the desired level. The brine should be circulated a few days after filling the tank to equalize the concentration of salt in the brine. The strength of the brine should be increased slowly until it is about 60° Salometer.

BRINING TECHNIQUE

The majority of picklers use the brine-salting technique for fermenting cucumbers rather than the dry-salting process just described. A "low" or a "high" salt-brining procedure may be used. The "low" salt brine has a salt concentration of about 30° Salometer, whereas the "high" salt brine has a salt concentration of about 40° Salometer. To start this process, brine of the desired concentration is placed in the bottom of the tank to a depth of about 1 ft to prevent damage to the cucumbers dumped into the tank. Dry salt and sufficient water may then be added to the cucumbers to obtain the desired brine strength, or the tanks may be filled with cucumbers and covered with brine of the desired concentration. This method, unlike the dry-salting process, does not depend on osmosis for water to fill the tank with brine.

When filled, the tanks are closed in the same manner as described for the dry-salting process. Circulation of the brine is desirable because it insures an equalized concentration of salt. Furthermore, it aids in more accurate control of the salt concentration in the brine. There probably are as many minor variations in the brining procedure as there are picklers. Regardless of the variations, it is mandatory to increase the salt concentration of the brine to at least 60° Salometer (approximately 15% salt). More detailed discussion of the brining procedures are given by Campbell,[11] Fabian,[32,34] Fabian and Bryan,[37] Fabian and Fulde,[39] Etchells and Jones,[29] and Jones.[51]

FERMENTATION OF SALT STOCK

The brined cucumbers undergo a "spontaneous" lactic acid fermentation. No attempt is made to control artificially the microbial population of the brine. The natural controls of the microbial population of the fermenting cucumbers are the concentration of salt in the brine, the temperature of the brine, the availability of fermentable substances, and the types and relative numbers of microorganisms present in the brine and on the cucumbers at the start of the fermentation. The fermentation proceeds slowly at first, but after 3 or 4 days the cucumbers generally are in full fermentation (Figure 23).

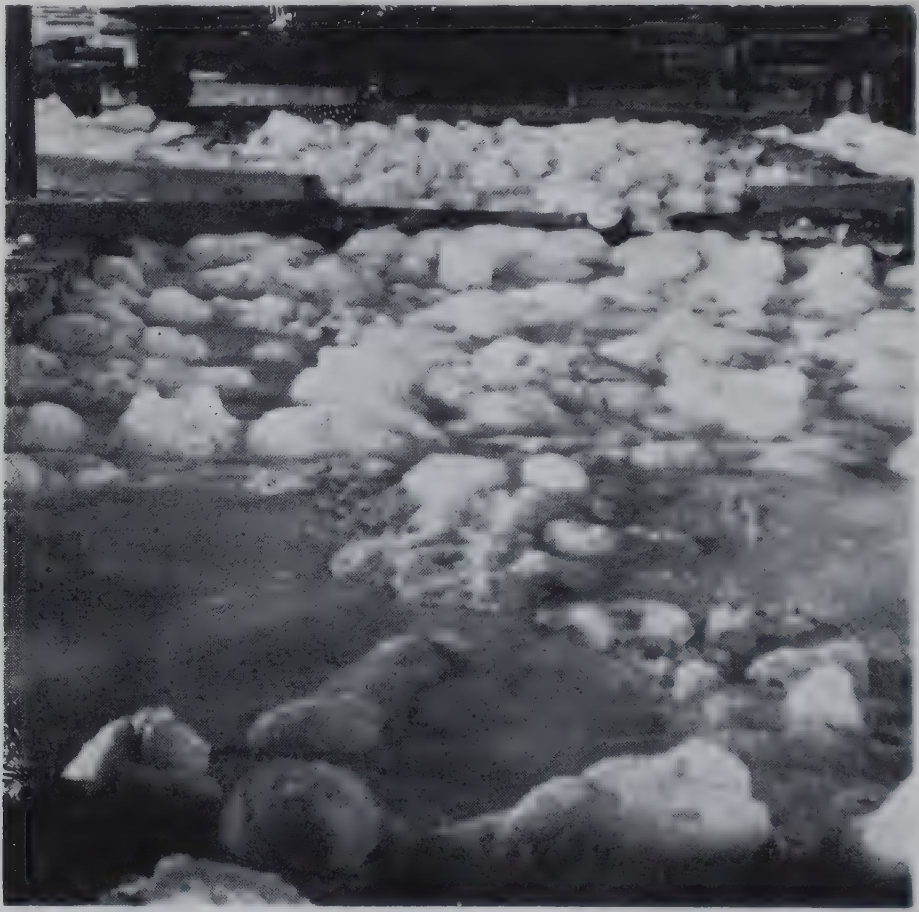


FIGURE 23. *Salt-Stock Cucumbers Undergoing Active Fermentation*

Besides the evolution of gas, which is one of the first visua manifestations of fermentation, there are other evidences of chang in the cucumbers and in the brine. The brine becomes cloudy takes on a straw-yellow color, and is effervescent because of th activity of the microorganisms. The color of the cucumbers changes as curing progresses, from the fresh pale-green color to a darke olive-green color. The white, opaque appearance of the flesh change to a translucent green. This color change proceeds from th periphery to the center so that the pickler can examine cross-section of the fermenting cucumbers and follow the rate of curing. When the cucumber has become completely translucent, curing is considered to be complete.

The chemical changes which occur in the brined cucumbers ar typical of a mixed fermentation.[52] Bacteria, yeasts, and sometime molds are responsible for the conversion of the fermentable substances present in the fresh cucumbers to gases, volatile and nonvolatile acids, alcohol, and traces of other end products. The desirable conversion of the fermentable matter is to the nonvolatile lactic acid which, together with the salt, preserves the pickles. Most of the lactic acid is formed by the lactic acid bacteria, although the coliform bacteria and others may also produce some lactic acid during the initial stage of fermentation. The lactic acid is formed in concentrations varying from about 0.5 to 1.0%.

Other products of fermentation which remain in some quantity in the salt stock are acetic acid and alcohol. The concentration of alcohol varies between 0.5 and 2.0% by volume. The acetic acid content is about 0.05 to 0.15%. Still other products, such as glycerol and mannitol, which may be formed generally are converted by the lactic acid bacteria and other microorganisms to other end products before completion of the fermentation. Therefore, if present, they are found only in traces, together with small quantities of acetylmethylcarbinol, esters, and other naturally occurring flavoring constituents that withstand the fermentation.

The gases have been identified as carbon dioxide and hydrogen. Hydrogen is formed only during the initial stage of fermentation, whereas carbon dioxide is produced as long as fermentable matter is present. Both gases are developed by the coliform bacteria, but only carbon dioxide is produced by the gas-forming lactic acid bacteria and yeasts.

As already stressed, the lactic acid bacteria are chiefly responsible for the desirable fermentation of cucumbers. However, there is a definite sequence of micoorganisms and microbial populations which occurs in both salt-stock and dill-pickle fermentations. In many respects, the population changes are similar to those for sauerkraut and olive fermentations.

The microbial sequence in normal fermentations of cucumbers (either salt stock or dills) may be divided into three stages: primary, intermediate, and final. During the primary or initial phase of fermentation, a great many unrelated bacteria, yeasts, and molds have been isolated. All of them are widely distributed in nature. At the beginning of this first stage of fermentation, the desirable lactic acid bacteria are far outnumbered by these other extraneous microorganisms. It is obvious, therefore, that the primary stage of fermentation is the most important phase of the pickling process During this critical period, if for any reason the fermentation doe not proceed in a normal fashion, any of the unessential microorganisms may predominate and contribute to undesirable deterioration of the cucumbers.

The primary stage normally requires 2 or 3 days, exceptionall as long as 7 days. During this time, the number of lactic aci bacteria increases rapidly, both fermenting and oxidizing types o yeasts become increasingly abundant, and the undesirable forms o bacteria decrease in number rapidly and may disappear entirel At the same time, there is a steady increase in total acidity and corresponding decrease in the pH of the brine.

A mixture of the low-acid-tolerant species of *Leuconostoc* an the high-acid-tolerant species of *Lactobacillus* are the predominatin bacteria in the intermediate phase of the fermentation. The undesirable bacteria have completely disappeared by the end of 10 t 14 days if the fermentation is normal. Yeasts are still present i significant numbers. The total acidity increases and the pH of th brine further decreases. The intermediate phase of fermentatio lasts for a variable length of time. During this period, however, th number of *Leuconostoc* types of bacteria increases and predominate but at the end starts to decrease rapidly and is replaced by specie of *Lactobacillus* as the acidity increases.

The final stage of fermentation is dominated by the aci tolerant lactobacilli which have replaced the species of *Leuconosto* The titratable acidity increases to a maximum (0.5 to 1.0%) an the pH decreases to a minimum of 3.5 to 3.8. However, once th fermentable substances have been exhausted and the species *Lactobacillus* can no longer produce lactic acid, the oxidative yeas begin to dominate the microbial population. As a result, the titratable acidity decreases and the pH value increases as the yeas oxidize more and more organic acids.

The common genera and species of bacteria found to predominate in the different stages of cucumber fermentations a shown in Table 42. It must be remembered that the salt concentration has a marked effect on the bacterial populations of cucumber fermentations. All of the bacteria listed will be found i cucumbers fermenting in 20° or 30° Salometer brine. However, the salt concentration increases, the relative abundance of bo species and total numbers will decrease until, at 60° Salometer, only the most salt-tolerant strains of *Lactobacillus* and the salt-tolerant yeasts will survive. This does not preclude the presence of halophilic microorganisms which, as far as is known, play no significant role in the fermentation.

TABLE 42. BACTERIA FOUND TO PREDOMINATE IN THE DIFFERENT STAGES OF CUCUMBER FERMENTATION

Stage of fermentation	Predominating species of bacteria
Primary	*Aerobacter aerogenes*[a,b]
	Aerobacter cloacae[a,b]
	Escherichia freundii[b]
	Escherichia intermedium[b]
	Bacillus mesentericus-Bacillus megatherium groups[c]
	Bacillus (Aerobacillus) polymyxa[d]
	Bacillus (Aerobacillus) macerans[d]
Intermediate	*Leuconostoc mesenteroides*[e]
	Lactobacillus plantarum[e,f]
	Lactobacillus brevis[e]
	Lactobacillus fermenti[e]
Final	*Lactobacillus plantarum*[e,f,g]
	Lactobacillus brevis[e]
	Lactobacillus fermenti[e]

[a] Etchells, Fabian, and Jones,[24] Etchells and Jones.[27]
[b] Unpublished data of Vaughn and Foda.
[c] Fabian and Johnson.[40]
[d] Unpublished data of Vaughn.
[e] Unpublished data of Vaughn and Tabachnick.
[f] Etchells and Jones.[30]
[g] Costilow and Fabian.[11a]

Yeasts are present throughout the fermentation and storage of salt-stock pickles. The presence and abundance of genera and species of these yeasts are influenced by the temperature, the relative amount of air, the salt concentration, and the presence or absence of sunlight. At low temperatures, the majority of the yeasts grows better than do most of the lactic acid bacteria found in the fermentation.

If the brine is frequently recirculated, it will carry enough occluded and absorbed oxygen from the air to allow yeasts to grow throughout the depths of the fermentation. Furthermore, since the salt-stock fermentations are conventionally conducted in open tanks, the yeasts very frequently dominate the fermentation, sometimes almost to the exclusion of the lactic acid bacteria.

The concentration of salt in the brine also influences the yeast population. At low concentrations (20° to 30° Salometer) more genera and species of yeasts are present. As the concentration o salt is increased, however, the number of yeast types able to grow i the brine is markedly reduced.

The presence of sunlight also considerably influences the yeas populations. Sunlight, because of its germicidal rays, prevents th growth of the oxidative, film-forming yeasts which grow on th surface of brines contained in sheltered or shaded tanks.

The genera of yeasts found in pickle brines in the Unite States include *Debaromyces, Pichia, Mycoderma, Torulopsis, Bre* *tanomyces, Zygosaccharomyces, Hansenula, Torulaspora,* an *Kloeckera.*[11a,22,23,23a,23b,67] The chief film-forming types are *Debar* *myces, Pichia, Hansenula,* and to a lesser extent *Mycoderma.* Th data of Etchells and Bell[23] indicate a sequence of types of yeasts. I is not known with certainty whether any of these yeasts play a important role in the fermentation or are detrimental to th production of the best salt-stock pickles. The author takes the vie that most of them are detrimental, because they consume the lact acid which, together with the salt, preserves the pickles for futu processing. It is certain that they are detrimental in the ferment tion of dill pickles and green olives, as will be discussed later.

STORAGE

After fermentation, the salt-stock pickles commonly are allowe to cure for 3 to 6 months before being used. Some manufacture will not use salt-stock pickles for making sweet pickles unless th are at least 1 year old. Salt-stock pickles may be stored in bri for several years if given adequate attention. Generally, the on controls consist of routine checking of the salt concentration an some effort to combat the film-forming yeasts.

The salt concentration must be increased to at least 6 Salometer for storage. The increase in salt concentration fro 40° to 60° Salometer is usually accomplished in about 1 mon after the tank is filled and headed.

If the salt-stock tanks are out of doors, the film-forming yeas do not present a serious problem. In sheltered or shaded tank however, these yeasts remain active and destroy the lactic aci They may reduce the acidity enough to allow the development undesirable microorganisms. Many picklers prefer to remove t film or membrane of yeasts (also called "scum" and "mycoderma") by skimming the surface of the brine regularly. As the yeasts cannot grow without an adequate supply of air, some prefer to use a layer of neutral mineral oil which is floated over the surface of the brine. The membrane also may be effectively controlled by use of ultraviolet radiation. Recently, sorbic acid (0.1% in the brine) has been recommended by Phillips and Mundt[78] for control of membrane formation. It appears to be more effective than acetic acid or mustard oil emulsions which also have been advocated.[7,21]

THE PROCESSING OF SALT-STOCK PICKLES

The removal of the excess salt from the pickles by leaching in water is termed "processing." Wooden tanks or vats equipped with steam pipes for heating the leaching water are commonly used for processing. The salt-stock pickles are placed in the processing tank, covered with cold water, and allowed to soak for 12 hours, or overnight. The leaching water is then changed and the pickles are allowed to soak for 6 to 8 hours more. The water is changed again and heated to 130°F (new, firm stock), or 150°F (old stock), and allowed to remain overnight. Alternative leaching methods include the use of four soakings in cold water; the use of three heated processing waters of 110°, 130°, and 140°F, respectively (the water being changed every 12 hours); or the use of water heated to 112°F, stirred at 10-minute intervals, and allowed to stand overnight. A steam siphon or pump is frequently used with the last method to keep the processing water in circulation. The following morning, the leaching water is removed, the pickles are flushed thoroughly with fresh water, covered with fresh, cold water, and sorted.

The same steps are taken in processing salt-stock pickles for sweet pickles as for sour pickles. Most picklers attempt to remove all of the salt during processing, so that on finishing, the concentration of salt can be more easily controlled.

Alum (potassium alum) and calcium chloride may be used during processing to firm and crisp the texture of the pickles. Alum is added at the rate of 1 lb per barrel (approximately 48 gal) of pickles. The alum is dissolved in warm water and added to the third processing water just before heating or, if the leaching process involves constant circulation of the water, it is added at the start

Calcium chloride is added at the rate of 1 lb per barrel, either t the third processing water, just before heating, or to the final swee liquor added to the pickles during the finishing process.

Sometimes when the salt-stock pickles are bleached near th blossom end, turmeric is used to restore some of the color. Turmeri gives the pickles a slightly yellowish appearance. It is added at th rate of 2 oz per barrel to the second processing water at the tim of heating or at the start of processing, if the water is circulate Turmeric, like alum and calcium chloride, must be listed on th label, if used.

After processing, the pickles may be sorted for size and qualit directly into barrels or kegs. The sizes, based on the number cucumbers per 45-gal cask, generally conform to those adopted b the National Pickle Packers Association.

THE FINISHING OF SALT-STOCK PICKLES

After processing and sorting, the pickles are *finished,* i.e., pr pared as sour, sweet, processed dills, mixed, mustard pickles, et

Sour Pickles. Preparation of sour pickles is very simpl The processed stock is placed in kegs or barrels and covered wit distilled vinegar. The containers are then sealed and, as soon osmotic equilibrium of the vinegar and the pickles has been reache the pickles are ready for shipment. An alternative procedure is "prime" the processed stock in distilled vinegar for a few days an then replace that vinegar with the final lot. At equilibrium th acidity of the pickle brine should vary between 2.0 and 3.5 acetic acid. Glass-packed sour pickles generally are less acid th those packed in bulk in barrels. Spiced sour pickles are made adding suitable mixed spices to the containers of pickles, or using spiced vinegar. Formulas for spicing sour and other kinds pickles are to be found in Campbell[11] or Joslyn and Cruess.[58]

Sweet Pickles. The procedure selected for making swe pickles depends on the quality desired, the plant capacity, and th time available for preparation. Sweet pickles are prone to shri and wrinkle badly if sugar is added too rapidly. Consequent every pickler has his own idea as to acidity, sugar content, spicin and step-wise procedure for finishing sweet pickles.

The most rapid method for finishing sweet pickles makes u of a spiced, priming vinegar to cover the pickles. The pickles a soaked in this vinegar for a day or two. Sugar is then added directly o the priming mixture twice a day for 7 to 10 days (depending on the rate of addition and the desired finishing concentration) to complete the process. In this procedure, the liquid is mixed at frequent intervals to prevent too rapid osmosis which otherwise will make the pickles shrivel. This method is designed for use with finishing tanks which hold large quantities of pickles.

The slow methods for finishing sweet pickles involve the usual priming or soaking of the pickles in distilled vinegar, generally in kegs or barrels. Then, instead of adding sugar directly to the priming vinegar mixture, this is drawn off and replaced with a sweetened vinegar. The mixture is then allowed to stand until the osmotic equilibrium has been reached. The liquor is again drawn off and replaced with sweetened vinegar which has been spiced. The mixture is allowed to stand as before. Then, if the sugar content has increased to a satisfactory level, the pickles are ready for shipment in bulk or for packing in glass. The acidity of finished sweet pickles should not be less than 2.0% as acetic. The sugar content may vary between 20 and 40%, according to the quality.

Sweet pickles are subject to attack by yeasts of the genus *Zygosaccharomyces*. These yeasts are osmoduric (sugar tolerant) or osmophilic (sugar loving). They may attack the sugar during the finishing process or cause fermentation in the finished pickles packed in glass, kegs, or barrels. Scrupulous sanitation is necessary to control them. Because they are so difficult to control, once the plant has been contaminated, many picklers resort to the use of sodium benzoate or pasteurization to destroy them in the finished product.[26,28,55]

Process or Imitation Dill Pickles. Process or imitation dill pickles are made by flavoring processed salt stock with a brine containing 5 to 6% salt and 3 to 4% acetic acid (distilled vinegar). It is desirable to flavor the brine with genuine dill brine and dill emulsion. It is common practice to place about 3 lb of pickled dill weed in the bottom of the barrel, a similar quantity in the middle of the half-filled barrel of pickles, and more dill weed and dill spices on top of the pickles in the filled barrel. The barrel is then headed and filled with the dill brine. The barrels are closed and stored for proper aging. After the aging period which is necessary for proper flavoring of the pickles, they may be used in bulk or packed in glass.

Mixed Pickles. Pickles, cauliflower, onions, green beans, green tomatoes, carrots, and peppers are sometimes used together to make mixed pickles and mixed pickle relishes. In general, these vegetables may be prepared in much the same manner as already described for salt-stock pickles. For more detailed discussion of the variations used for pickling vegetables other than cucumbers as well as for pickling fresh cucumbers, other fresh vegetables, and fruits, the publications of Campbell,[11] Cruess,[17] Fabian and Blum,[30] Etchells and Jones,[29] and Joslyn and Cruess[58] should be consulted

Fermented Dill Pickles

Genuine dill pickles differ from the other well-known cucumber pickles because they are the product of bacterial fermentation in spiced salt brine. They owe their distinctive flavor and aroma to the products of fermentation of the lactic acid bacteria and to the blending of dill herb and spices which are added to the brine.

The larger cucumbers are generally used for preparing fermented dill pickles. The fresh cucumbers are washed and placed in 45 to 50-gal barrels with pickled dill weed and dill spices as described for process dills. Some picklers add dry salt with the dill herb and other spices and then fill the headed barrel of cucumbers with water. Others prefer to use a salt brine (5 to 7% sodium chloride) which may or may not be spiced with dill emulsion. Many picklers also add 2 to 3 qt of vinegar per barrel of pickles to help retard the growth of undesirable microorganisms, by decreasing the pH value of the brine.

One formula given by Campbell[11] for spicing dill pickles is as follows:

Drained dill weed (cured in vinegar and salt)	8 lb
Dill vinegar from barreled dill weed	3 qt
Mixed dill spices	3 pt
Salt	16 lb

Enough cucumbers to fill the barrel. Potable water to cover the contents and completely fill the barrel.

Some dill pickles are heavily seasoned with dried cayenne peppers; others may be flavored with garlic, caraway seed, anise, o ennel to make specially seasoned dill pickles for certain trade preferences. Sweetened dill pickles may also be produced.

The filled barrels may be bunged tightly to exclude air; or small holes may be drilled through the bungs to allow for the escape of fermentation gases. Some omit the bungs during the period of active fermentation. Whatever the procedure, it is mandatory to keep the barrels full of brine at all times because the exposed pickles will spoil rapidly. The salt concentration in the brine should be maintained at 20° to 25° Salometer. Optimum temperature for the fermentation is between 70° and 80°F. The barrels may be stacked indoors or allowed to ferment outdoors, as shown in Figure 24. The fermentation is usually active for 3 to 4 weeks after which an additional curing period of 3 to 4 weeks is considered essential. The flesh of dill pickles becomes entirely translucent when completely cured. The brine has about 0.5 to 1.2% total acidity calculated as lactic acid. In addition, there is a small amount of volatile acid (about 0.2% calculated as acetic) and alcohol (about 1.0% by volume) produced by fermentation.[56]

As already mentioned, the desirable fermentation of dill pickles is caused by the lactic acid bacteria. There is usually a definite

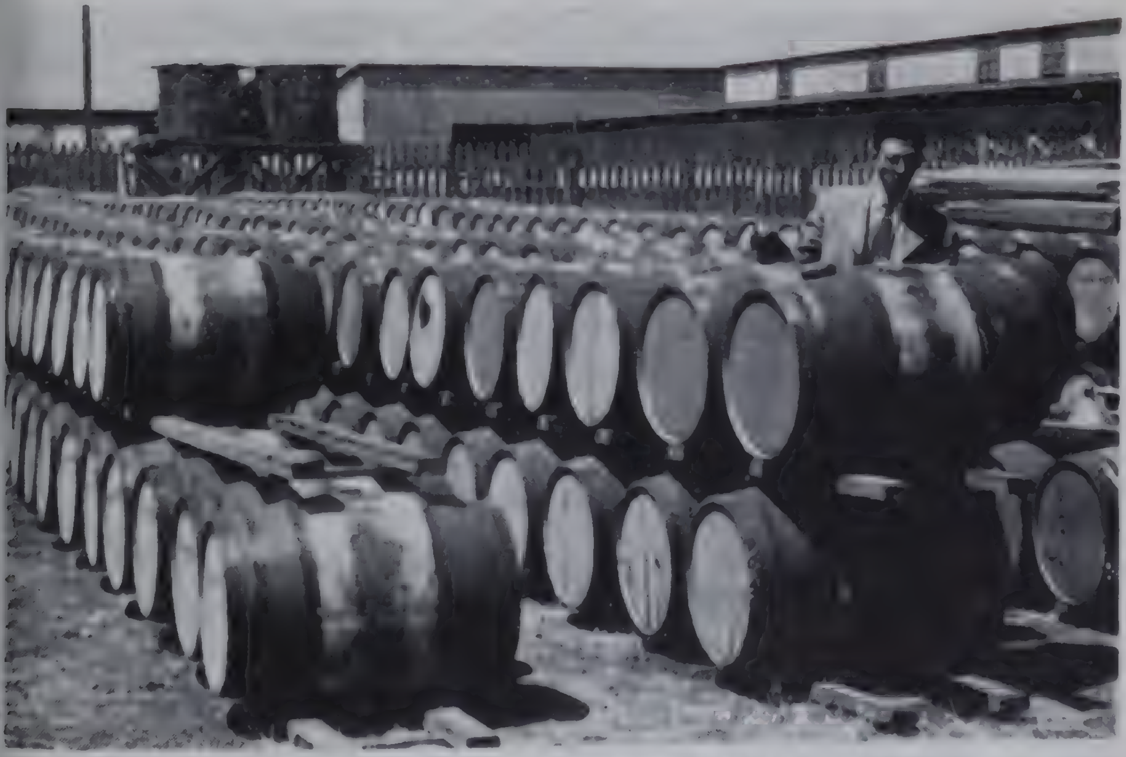


FIGURE 24. *Barrels of Fermenting Dill Pickles in a Fermentation Yard*

sequence of bacterial types. During the early stage of fermentatior the coliform bacteria and aerobic spore-forming bacteria may pre dominate. Soon, however, the Gram-positive cocci of the genu *Leuconostoc* gain the ascendancy. These in turn are eclipsed by th Gram-positive rods of the genus *Lactobacillus*. All of the bacteri listed in Table 42 have been recovered from fermenting dill pickle by the author and his students.

Yeasts may also be present, but if they are present in appre ciable numbers they have an undesirable effect on the fermentatio The yeasts compete with the lactic acid bacteria for fermentabl carbohydrates and besides may oxidize the lactic acid formed in th fermentation. The dissipation of both fermentable sugars and lact acid by the yeasts decreases the total acidity and increases the pl of the brine. Thus the yeasts may contribute to spoilage of th pickles by making conditions favorable for the growth of oth undesirable microorganisms.

Overnight Dill Pickles

"Overnight" dill pickles are somewhat similar to genuine di pickles, with the important exception that they are stored at 38° where a slow lactic acid fermentation produces an acidity of on 0.3 to 0.6%, calculated as lactic at the end of 6 months. "Overnight dill pickles retain some of the fresh cucumber flavor, but are s perishable that they are being replaced by unfermented, pasteurize dill pickles made from freshly harvested cucumbers.[26,32]

Deterioration of Pickles

Extensive study has been made of the deterioration of cucum bers during fermentation, curing, and storage.[31] For the most par deterioration of pickles is caused by microorganisms. Chemic deterioration generally is confined to metallic contamination or un anticipated alteration of flavor and aroma by the use of specif chemicals or undefined congenerics used for spicing purposes.

The role of yeasts in the deterioration of pickles has alrea been discussed. The most important defect caused by both bacter and molds is softening. Gaseous deterioration or "floater" spoila is another common spoilage caused, for the most part, by bacteri A comparatively rare deterioration, in California at least, is th formation of dark (black) brines and pickles caused by the activi f certain bacteria. Other spoilage of infrequent occurrence is the utyric fermentation and formation of "ropy" brine.

OFTENING

Softening is a progressive spoilage which occurs most frequently oon after the brining of dill pickles or salt-stock pickles brined in ess than 30° Salometer brine. The skin of the cucumber is attacked first, usually in spots at the blossom end. In a short time, the entire skin may be affected, become slippery, and be easily removed. This characteristic manifestation of softening has given rise to the terms "slips" or "slippery" pickles in the industry. "Mushy" pickles result when softening involves the deeper layers of cells in the pickles.

Most of the softening of commercially processed pickles results from the activity of microbial enzymes which attack the pectic substances present in the middle lamella separating the individual cells of the cucumber. Kossowicz[59] was probably the first to discover the importance of *Bacillus mesentericus* and closely related species of aerobic, spore-forming bacteria as agents of cucumber-pickle softening. His work has been confirmed by Rahn,[81] LeFevre,[60,61] Joslyn,[57] and more recently by Fabian and Johnson.[40] The latter investigators made a study of the purely chemical and physical types of softening as well as of softening caused by bacteria.

On the basis of all of these investigations, it may be concluded that most of the microbial softening is caused by members of the *Bacillus mesentericus-Bacillus megatherium* group of bacteria. According to the author's own unpublished experience, however, other microorganisms which may also cause softening of pickles include bacteria of the *Bacillus (Aerobacillus) macerans-Bacillus polymyxa* group, and molds of the genus *Penicillium*. Softening occurs when microorganisms are capable of elaborating pectin-destroying enzymes (see Phaff and Joslyn[77] and Kertész[58a] for detailed discussions of pectic enzymes) under the conditions of salinity, acidity, etc., which exist in pickle brines. It appears that the pectolytic enzymes produced by bacteria are most active in the alkaline range, i.e., above pH 7.0 and are rapidly inactivated in acid surroundings (see Wood,[94] Kraght and Starr,[59a] and Nortje and Vaughn[69a]). The reverse is true of the fungal pectolytic enzymes, according to the work of Ehrlich,[21a] Waksman and Allen,[90a] Phaff,[76a] Luh and Phaff,[64a] Pavgi, Pithawala, Savur, and Sreenivasan,[70a] and others.

Recently Bell and coworkers[4a,5a] have demonstrated the presenc of the enzyme pectinesterase in the seeds, leaves, petioles, stem flowers, and fruit of the pickling cucumber. The role this enzym may play in softening of fermenting cucumbers is not clear. Thu it is possible that the pectic enzymes elaborated by bacteria, mold yeasts, and perhaps the intrinsic enzymes of the cucumber itse may be involved in softening. At present, it is not possible to stat clearly whether one or a combination of these agents causes mo of the softening.

Softening may also be induced by purely chemical or physic changes not involving microorganisms. Thus, Lesley and Cruess concluded that softening might, in certain instances, result fro acid hydrolysis of pectic substances, particularly in immature c cumbers. Fabian and Johnson[40] confirmed this conclusion and the same time showed that cooking also contributed to softening pickles. It is to be stressed, however, that neither use of stron mineral acids (HCl to pH 2.2) nor cooking are integral steps in th production of salt-stock or dill pickles.

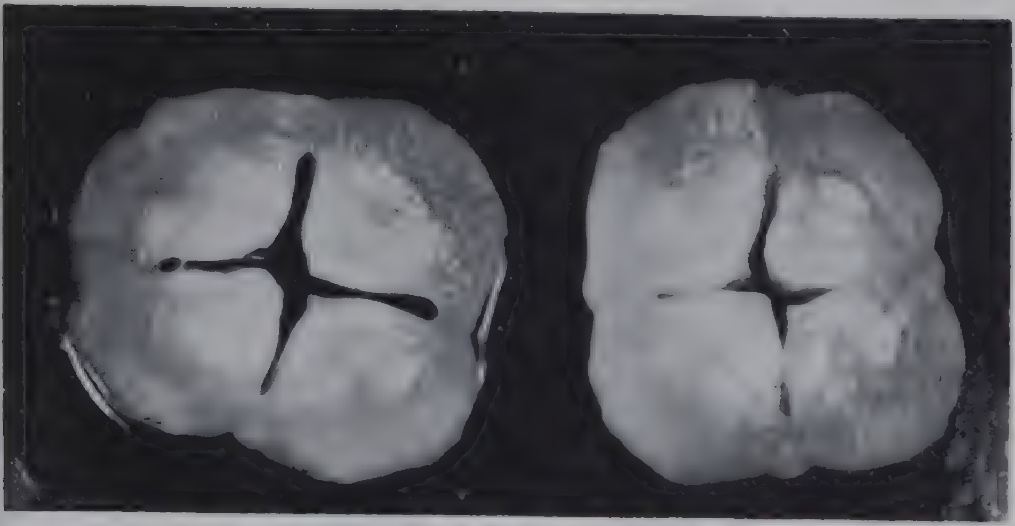
Faville and Fabian[44] have suggested that bacteriophages antibiotic substances antagonistic to the lactic acid bacteria ma contribute to softening of cucumbers by favoring the growth the spoilage bacteria. It is unfortunate that their *in vitro* studi were not substantiated by comparable *in vivo* tests.

The recently developed polygalacturonase enzyme test of Bel Etchells, and Jones,[5] if applied as suggested, should permit ear detection of potentially undesirable softening of fermentin cucumbers.

Gaseous Deterioration

Cucumbers frequently undergo a type of deterioration whic causes them to become so distended from gas pressure, that th tissue is permanently distorted and large gas-pockets form (Figu 25). Such pickles have an unnatural puffed or bloated appearan and, if the hollow centers are filled with gases they will float. Suc pickles are most commonly called "bloaters" or "floaters."

Hollow pickles have been known to occur for a long tim Many possible explanations for their formation have been set fort at one time or another: the wrong variety of cucumbers; too rap fermentation; insufficient weighting and too loose packing of th



IGURE 25. *Cross-Section of Salt-Stock Pickle to Show Rupture of Flesh Resulting from Gas Production*

ucumbers in the tanks; insufficient salt in brine; and too large ucumbers. LeFevre[61] was one of the first to observe that "floaters" vere hollow and most frequently occurred in the larger cucumbers ickled in brines containing too little salt. However, until very ecently, the cause of their formation was largely conjectural.

Although it was suspected earlier, the first substantial evidence hat gaseous deterioration was caused by microorganisms was pre- ented by Veldhuis and Etchells[90] in 1939. These investigators ound that hydrogen was produced in significant quantities in the ickle fermentations in 60° Salometer brine and in some, but not all, ermentations at lower salt concentrations. They also found that as from the hollow cucumbers from 60° Salometer brines contained ssentially the same amount of hydrogen and about the same ratio f hydrogen to carbon dioxide as the gas collected from the surface f the fermentations. They also isolated, but did not identify, an rganism which produced significant quantities of hydrogen. Some- vhat later (1941) Jones, Etchells, Veerhoff, and Veldhuis,[54] and tchells and Jones[25] suggested that gaseous fermentation by un- dentified yeasts was the cause of "floater" formation. Still later 1945) Etchells, Fabian, and Jones[24] presented evidence that coli- orm bacteria of the genus *Aerobacter* were responsible for the ormation of hydrogen in pickle fermentations.

"Floater" spoilage can be minimized under commercial con- itions by elimination of the largest sizes of cucumbers; by the use f a needling machine to puncture the cucumbers and thus provide for escape of gases during the fermentation; by rigid control o the fermentation by trained personnel; and by scrupulous sanitatio

BLACK BRINE AND PICKLES

This form of deterioration is characterized by the formatio of dark brine (deep brown to black) and the occurrence of darkene areas on the outside of the pickles. The blackening starts in th upper layers of the brine and extends downward. Hydrogen sulfid or a definite putrid odor is characteristic of this spoilage.

Rahn[81] was one of the first to investigate the problem of blac pickles. He found that the blackening resulted from the productio of iron sulfide. The bacteria present in the brine caused the r duction of gypsum ($CaSO_4$). The hydrogen sulfide thus forme reacted with iron present in the brine to give iron sulfide. Fabia Bryan, and Etchells[38] confirmed and extended the work of Rah to include a discussion of the possible liberation of hydrogen sulfi in the course of protein breakdown by bacteria. An addition source of blackening was found by Fabian and Nienhaus[42] to resu from the production of a black, water-soluble pigment by *Bacill nigrificans.* According to Fabian and his students, blackening ca be prevented by controlled fermentation which insures a significa increase in acidity of the brine and elimination of iron and sulfa contamination.

In all probability, black brine in pickle fermentation, inste of always involving sulfate or protein reduction, frequently is t result of the reaction of iron with complex tannin compounds form complex iron tannates which are black, bluish black, or gree ish black. Iron tannates will not form unless the iron is oxidized the ferric state. Iron sulfide, however, will also form from ferro iron, so that the blackening of pickle brines should be nearly u form throughout instead of starting at the surface and extendi downward.

It is also possible that some of the darkening of pickles, pa ticularly sweet ones, may result from nonenzymic chemical reactio not involving iron, tannins, or hydrogen sulfide (Stadtman[8

ROPY BRINE

Ropy brine is another defect which appears during the f mentation of pickles. Fabian and Nienhaus[42] have investigat ne problem and advocate the control of slimy or ropy pickle rines by rapid increase in the salt content of the brine. However, nless the slimy or ropy brine has a deleterious effect on the uality of the finished pickles it should not be considered as a deect. Many of the bacteria known to produce polysaccharides (dextrans, levulans, etc.) are found in normally fermenting ickles; *Leuconostoc mesenteroides,* in particular, is usually presnt. Furthermore, other bacteria and yeasts which normally occur n the pickle fermentations will, in time, probably utilize the olysaccharides.

HEMICAL DETERIORATION

The chief cause of chemical deterioration of pickles is the irect addition (wittingly or unwittingly) of undesirable chemicals o the brines or to the pickles themselves. The changes which occur nay affect the appearance or the flavor and aroma of the pickles.

Contamination with copper and iron is probably the most ommon type of chemical deterioration. Vinegar or lactic acid hich may be used for preparation of brines or liquors for pickles nay be heavily contaminated with copper and iron (see Chapter 7 of Volume 1). Other copper and iron contamination may result rom contact of the comparatively corrosive brines used for pickling vith corrodible equipment made with alloys which contain copper nd iron.

Iron, as already described, is involved in the blackening of ickle brines and pickles. Copper replaces magnesium in chlorophyll contained in the pickles and causes them to turn an unnatural, rtificial green color. Only 5 to 10 ppm of copper in the brine or iquor is enough to spoil the pickles. Both copper and iron may ause a metallic flavor.

Other possible sources of chemical deterioration include contamination of cucumbers with chemical sprays, etc., used to combat nsects and microorganisms in the fields. Some of these compounds nay remain on the cucumbers and cause off-flavors and off-odors in he pickles.

A comparatively new source of deterioration is the too generous se of "spice oils" for flavoring pickles. "Spice oils" first were ecommended by Fabian[33,35,43] and others,[49] because many whole spices were too heavily contaminated with undesirable bacteria.[41] Methods were developed which insure the availability of co mercially sterile spices and other flavoring agents. For reasons economy, it is still thought desirable to increase the use of "sp oils." However, if these preparations are not manufactured c rectly and are not used properly by the pickler, undesirable med inal flavors and tastes result.

Packaging Finished Pickles

Formerly, almost all pickles were sold in bulk from wood kegs, barrels, casks, etc., but now glass and sometimes tin wooden containers are also used. Pickles do not keep very long wooden containers. Therefore, care must be taken to provide c storage (50°F) and to protect against drying out of the barrels freezing the contents. The wooden containers should be inspec at weekly intervals and all broken or leaky ones should be repair All lost liquor must be replaced to prevent darkening and softeni of exposed pickles.

Pickles may also be packed in tin cans. The high acidity the pickle products, however, makes prevention of can corrosio major problem. The tin plate must be heavily lacquered or coa with paraffin to retard can corrosion. Pickles have been m commonly canned in No. 2½, No. 10, and "full gallon" cans. canning, the pickles are hand-packed into the cans and cove with dill brine, vinegar, or spiced sweet vinegar, according to type of pack. Pickles canned in lacquered cans are exhaus to insure a suitable vacuum in the cooled, closed container. prevent undue softening, canned pickles should be cooled imme ately after heating. Exhausting and cooking are impossible paraffined cans are used. Pickles packed in cans will keep somew longer than those packed in barrels or kegs, if corrosion is retard

Glass-packed pickles are packed by hand into jars which filled with liquor of the appropriate type and exhausted in so manner before closing to insure a vacuum in the container, or closed without a vacuum. Entirely mechanized lines, similar those used with cans, are available for handling the jars, o they have been packed with pickles. Pickles packed in glass keep much longer than either canned or bulk-packed pickles they have been properly packaged and stored.

THE PRODUCTION OF SAUERKRAUT

The use of cabbage (*Brassica oleracea*) as a food by man, as ell as the use of cucumbers and olives, probably antedates recorded istory. According to Magruder,[65] cabbage has been used rather ommonly for about 4,000 years and, next to potatoes, is the most ommon vegetable in the diet of man.

Sauerkraut, a food resulting from the lactic acid fermentation of cabbage, to quote Prescott and Proctor,[80] "has been a popular ood in Europe for at least 4,000 years, and probably has as its ntecedent the somewhat similar preserved cabbage soaked in sour wine or vinegar which was popular even before the Christian era."

Originally sauerkraut was made only in the home because t provided a means of preserving fresh cabbage which might otherwise spoil before it could be used. However, the commercial production of sauerkraut has become an important food industry in he United States.[17,62,79,80] Because the major part of the industry s localized in those states bordering on the Great Lakes, sauerkraut manufacture is a seasonal industry which usually works from Sep-ember through December. Cabbage varieties best suited for growth n these regions are used.

The varieties grown include Early Flat Dutch, Late Flat Dutch, Early Jersey Wakefield, Early Winningstadt, Glory of Enkhuizen, Copenhagen Market, Marion Market, Globe, Danish Ballhead, or pecial varieties and strains bred from them. When the cabbage is harvested, care is taken to select only the best heads and to prevent undue injury because only sound, firm, compact, uncolored cabbage hould be used for sauerkraut. Immature and partially spoiled heads, if used, will result in an inferior product.

Preparation for Fermentation

The harvested heads are stored to allow the outer leaves to wilt before the sorting, trimming, and cutting operations are started. Storage may take place in piles outside the plant or in special bins or rooms where the temperature and air supply can be controlled to he best advantage. Controlled storage allows the maintenance of a uniform temperature of at least 70°F and the trimming and hredding of thus wilted cabbage with the least breaking and tear-ng of the leaves.

When the cabbage has been wilted sufficiently, it is passed b conveyor belts to workers who remove the wilted outer green leaves sort and cut out any bad spots, and then core each head by machin There are two types of coring machines. One removes the cor entirely. The other, more common type, cuts the core into fin pieces without removing it from the head of cabbage. In the firs case, the core is discarded; in the second case, the finely cut cor is converted into sauerkraut. Alternatively, the wilted cabbag may be cored by machine and then passed through a trimming an washing machine before it is shredded.

The trimmed and cored cabbage then passes by conveyor to th cutters, where it is shredded. The cutters are power-driven, rotar adjustable knives, capable of cutting the cabbage into long shre as fine as 1/16 to 1/32 inch in thickness. In general, long, finely cu shreds are preferred, but the thickness is determined by the jud ment of the manufacturer. The shredded cabbage (known as slaw is then conveyed by belts or by hand-carts to the vats for salting an fermentation (Figure 26).

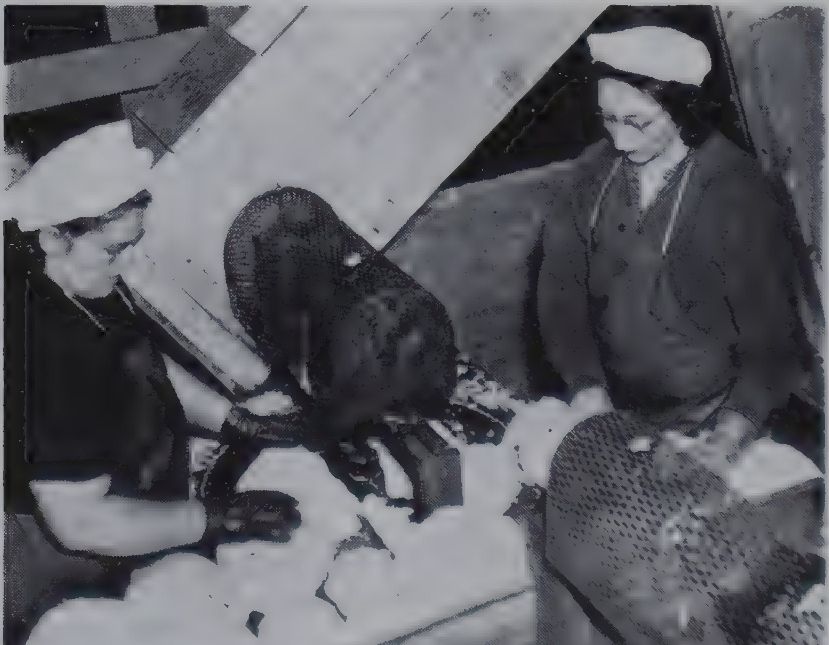


FIGURE 26. *Slicing Fresh Cabbage for Fermentation* (Courtes National Kraut Packers Association)

Salt plays a very important role in the making of sauerkra and the concentrations used are rigidly controlled. According legal definition,[86] the concentration of salt must not be less th

2%, nor more than 3%. As a result, most producers use a concentraion of about 2.5% of salt (2.5 lb of NaCl per 100 lb of cabbage). Salt is required for several reasons. It extracts water from the shredded cabbage by osmosis, thus forming brine. It suppresses the growth of some undesirable bacteria which might cause deterioration of the product while making conditions favorable for the desirable lactic acid bacteria which cause fermentation. Salt also contributes to the flavor of the finished sauerkraut.

It is essential that the salt is uniformly distributed throughout the mass of shredded cabbage. For this reason, some producers prefer to salt the shredded cabbage in the hand-carts which convey it to the fermentation vats. Uniform mixing is much more certain under such conditions. Others salt the cabbage as it is packed into the vats.

The vats should be filled carefully, to make certain that the cabbage is evenly distributed and packed as solidly as possible. The vats are filled to about 1 ft above the top, the slaw is covered with a thick layer of clean, outer leaves, and then is fitted with a heavy cover which is heavily weighted. The additional weighting is necessary to facilitate formation of the brine and to assure that the brine will completely cover the cabbage at all times. Unless this is done, the top layers of sauerkraut will have to be discarded when the vat is emptied, because they may have become soft and discolored or otherwise deteriorated. In extreme cases, all of the kraut may have to be discarded.

Within a few hours, the brine has formed and the fermentation has started. The head is then fixed in position in much the same manner as with pickle tanks. Within 3 or 4 days, the fermentation has become very active and the acidity increases rapidly. Under normal conditions of temperature, the fermentation is usually complete within 3 or 4 weeks.

The Fermentation

The normal fermentation of sauerkraut is a spontaneous lactic acid fermentation caused by a natural mixture of lactic acid bacteria. The fermentation proceeds rapidly at temperatures between 60° and 75°F and generally some provision is made to keep the temperature within this range. Temperatures much below 60°F result in a slow fermentation which may not be completed until the following spring. Temperatures above 75°F may result in a ver rapid fermentation, but opinion is divided concerning the qualit of kraut produced at temperatures of 85°F and above. At tempera tures of 75° to 85°F, the final acidity is usually attained withi 2 to 3 weeks, whereas at lower temperatures, the fermentation ma be prolonged to 4 or more weeks.

The sauerkraut fermentation normally proceeds more rapidl than either the pickle or olive fermentations. Fresh cabbage ger erally contains more sugar (about 2.9 to 6.4%, according to Atwate and Bryant[2] and Peterson et al[75,76]). The sugar is more readil available because the cabbage is shredded. Furthermore, the fe mentation occurs in the presence of much less salt.

The chemical changes which occur in the sauerkraut ferment tion are typical of a mixed fermentation caused by bacteria an yeasts. A variety of microorganisms is responsible for the conversic of the fermentable sugars present in the shredded cabbage to gas (carbon dioxide and hydrogen), volatile and nonvolatile acid alcohol, and traces of other end products.[73] The major end produc of the sauerkraut fermentation are lactic acid, acetic acid, alcohc and carbon dioxide. Small quantities of hydrogen may be produce by the coliform bacteria during the early stages of fermentatio Glycerol and mannitol may also be formed, but they usually a found only in traces in the completely fermented kraut, togeth with quantities of flavoring constituents, which may be of natur occurrence or result from the fermentation.

Lactic acid bacteria are mainly responsible for the normal fe mentation of sauerkraut. A definite sequence of microorganism and microbial populations is known to occur (Pederson[71,72a,72b,74 The sauerkraut fermentation may also be divided into three stage primary, intermediate, and final. At the beginning of the ferment tion, the desirable lactic acid bacteria are outnumbered by a gre many unrelated bacteria, yeasts, and molds. During the prima stage of fermentation, the desirable lactic acid bacteria begin develop very rapidly and, by the end of 2 or 3 days, the g producing cocci dominate the population during the intermedia stage.

During this period, the total acidity has increased very rapid to a 0.6 to 0.8% total acidity, calculated as grams of lactic acid p 100 ml of brine. After 4 to 6 days of fermentation, the rod-shap lactic acid bacteria begin to dominate the population. During th final stage, the gas-forming (heterofermentative) and non-gas-forming (homofermentative) species of *Lactobacillus* are active. However, the homofermentative species probably complete the fermentation, because they dominate the *Lactobacillus* population, according to Pederson.[71]

The sequence of microorganisms observed in the fermentation of sauerkraut is essentially the same as that found in the fermentation of cucumbers in low-salt brines (see Table 42). However, the rate of acid production in cabbage is significantly faster than in either cucumbers or olives, because the low concentration of salt and readily available fermentable sugar favor the development of much larger total population levels of the desirable lactic acid bacteria in the sauerkraut. Furthermore, the sauerkraut fermentation is comparatively anaerobic. Therefore, fermentable substances are not dissipated by the yeasts to the extent they are in tanks of salt-stock pickles or olives.

Completely fermented sauerkraut must contain "not less than 1.5% of acid, expressed as lactic acid."[86] In reality, most of the completely fermented sauerkraut contains more than the required minimum of 1.5% of acid. The final acidity may range up to almost 2.0% as lactic acid. The total acidity is composed of lactic acid and acetic acid; the ratio of lactic acid to acetic acid varies from about 4:1 to 6:1. The finished kraut also contains alcohol in concentrations varying from about 0.2 to 0.8% by volume.

Storage, Processing and Packing

It is not customary to keep sauerkraut for more than one season, as is sometimes done with salt-stock pickles. As soon as the kraut has been completely fermented, it is ready for canning or shipment in bulk in wooden kegs or barrels. The first sauerkraut made in the fall is usually canned or shipped in bulk within a short time after the fermentation is completed. Frequently, a tank is used for fermentation two or more times in a season. However, it is necessary to store some of the sauerkraut for as long as 6 to 8 months under certain circumstances. It may be stored in kegs or barrels or in the original fermentation tanks. It must be covered with brine and kept from contact with air at all times. Storage in sealed wooden containers provides good anaerobic conditions, but is not economical. Tank storage is commonly used.

When the fermentation is completed, the top layer of kraut, protecting cabbage leaves, and brine is cleared of all foam and microbial film. If the brine is low, more is added to cover the shreds. Then the shreds and juice may be covered with a muslin cloth to aid in keeping the top layer of kraut moist. The cloth is then covered with the wooden tank cover which is set in place as before. An alternative method is to flood the top of the sauerkraut and brine with a layer of neutral mineral oil to provide anaerobic conditions and then reset the cover.

PROCESSING

The vat of sauerkraut is opened by removing the weights and anchoring the timbers used to keep the wooden cover in place. All liquid above the cover is withdrawn. Then the cover is removed. If the sauerkraut has been kept well covered with brine, there is little loss, but if the brine has been allowed to recede below the surface of the shreds, the discolored and otherwise deteriorated product must be discarded until sound sauerkraut has been reached. Then, the plug in the bottom of the tank is opened to draw off the juice ahead of the removal of the sauerkraut for repacking in bulk or canning.

PACKING IN BULK

Bulk distribution of sauerkraut is accomplished by repacking the kraut in kegs or barrels. The wooden container is filled with "dry pack" sauerkraut, consisting of about 90% solids and 10% brine. Because of the perishable nature of sauerkraut, bulk distribution is limited primarily to the cooler months.

CANNING

Sauerkraut is relatively simple to can. It is a very acid food and thus does not require prolonged cooking under pressure to preserve it. It is easily canned with little equipment.

The kraut is removed from the tanks with forks and passed to the canning tables in large pans or other containers or by means of conveyor belts.

In the simplest canning operation, the compacted mass of shreds is thoroughly broken apart by hand, allowed to drain, hand packed into cans (Figure 27), brined, and then exhausted to insure heating the kraut in the center of the can to 150°F or more. In this type of operation, the filling of the cans may be expedited by use of the rotary hand-pack filling machine.

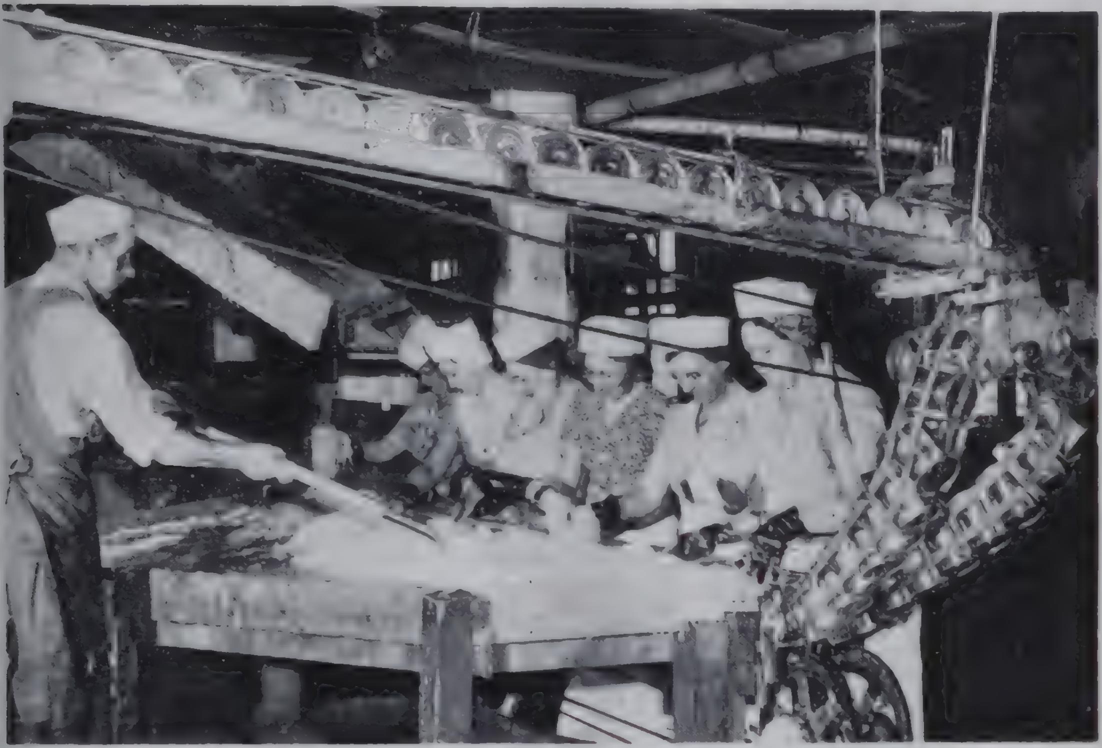


FIGURE 27. *Hand Packing Sauerkraut into Cans* (Courtesy—National Kraut Packers Association)

Some packers prefer to heat the sauerkraut before filling it into cans. In this method, the compacted kraut is first broken apart by hand and then heated batchwise in its own brine at 110° to 120°F. The warm sauerkraut is then passed to the canning line for the conventional hand filling, brining, exhausting, closing, cooking, and cooling. If the sauerkraut has been warmed in this manner, hot brine can be added and very little subsequent exhausting is necessary.

Much labor savings are possible if special kraut filling machines are used. By this method, the cans may be filled with sauerkraut and brine hot enough to make subsequent exhausting and cooking unnecessary. However, most prefer to use a short cook as an added protection.

Irrespective of the method, the center temperature of the can of sauerkraut with brine should be about 150°F at the time of closing for an adequate exhaust.

Although sauerkraut is a very acid food product, it is believed necessary to cook (process) the cans at 212°F in order to insur commercial sterility and prevent microbial spoilage of the canne product. Processing is necessary because of the very irregular he penetration resulting from clumps of heavily compacted shre which are not broken apart well before canning. According t Bitting,[6] the cooks for sauerkraut should be 10 minutes at 190° for No. 2½ cans and 15 minutes for No. 10 cans in the agitatin cooker, or 35 minutes at 212°F for No. 2½ cans and 45 to 50 minut for No. 10 cans in stationary cookers. Campbell[11] points out, hov ever, that if the filled cans are closed with a center temperature over 160°F, it should not be necessary to cook the kraut. Accor ing to Cruess,[17] canned sauerkraut should be processed in stea long enough for the contents of the can to reach 180°F.

It is very desirable to cool the canned sauerkraut thorough after cooking. Judicious cooling preserves the color, prevents cook tastes and undue softening of the shreds.

Defects of Sauerkraut

There are several rather common defects of sauerkraut whic have been recognized. Some of these are of microbial origin; othe apparently are chemical in nature. Discoloration of sauerkraut, o of the most common defects, may be caused by chemical reactio or by the activity of microorganisms. Softened, slimy, or rott krauts, according to the literature, are largely of microbial origi Off-flavored kraut results either from the activity of microorganis or from direct chemical contamination.

Darkening of sauerkraut probably is the most common defe If the fermenting kraut is not covered and weighted properly, t brine may fail to cover or moisten the upper layer of shre Darkening will result either by direct chemical oxidation or the growth of aerobic microorganisms. Excessive amounts of ir may react with tannins from the wooden tanks or covers or sulfid may be produced by fermentation to cause black discoloratio Uneven distribution of salt or use of too much salt may disrupt t normal fermentation and allow undesirable microorganisms to pr duce discoloration.

Pink sauerkraut is caused by the activity of aerobic yeas Since the yeasts grow only in air, they usually develop in the bri at the surface of the tank of sauerkraut, on the exposed shreds, n the air pockets formed between the sides of the vat and the hredded product. The yeasts produce a pigment that varies from a light pink to an intense red color. They are undesirable because they utilize sugar and acid, in addition to causing discoloration. According to the literature, any inhibition of normal fermentation may favor the development of the pink yeasts. (See Brunkow, Peterson, and Fred,[9] Fred and Peterson,[46] Marten, Peterson, Fred, and Vaughn,[66] Pederson,[72] Pederson and Kelley,[74] Peterson, Parmele, and Fred.[76])

Soft kraut is usually associated with the presence of air pockets, faulty salting, high temperatures, or abnormal fermentation (Fabian[32]). Nevertheless, it should be stressed that although it is thought that softening is caused by microorganisms, their identity is unknown.

It is claimed in the literature that slimy or ropy sauerkraut is the result of the growth of *Lactobacillus cucumeris* and *Lactobacillus plantarum*. (The name *L. cucumeris* is regarded by most authorities as a synonym of *L. plantarum*.) It is not likely that these homofermentative lactobacilli are involved in the production of slimy or ropy kraut. Neither of them is known to produce polysaccharides. Furthermore, they occur in the normal fermentation of sauerkraut.

Rotting of sauerkraut is claimed to be caused by the presence of certain types of bacteria, yeasts, molds, and even fruit flies. Apparently, cases of rotting occur only when the sauerkraut is seriously neglected. Adequate protection of the surface layers of the sauerkraut will prevent rotting. However, it should be stressed that if rotting has progressed to such an extent that insects have infested the sauerkraut, better plant sanitation is essential.

Off-flavors in sauerkraut have been demonstrated by Pederson[71] and others to result from changes in the sequence of types of desirable lactic acid bacteria in the fermentation. For this reason, starters of pure cultures of some of the lactobacilli are not recommended. Off-flavors may also result from the activity of undesirable microorganisms which cause softening or rotting of sauerkraut. In some cases, chemical contamination resulting from improper use of sterilizing agents, insecticides, etc., may produce off-flavors.

Spoilage of canned sauerkraut results from understerilization or from can corrosion. Either type of spoilage is due to development of gas which causes the cans to swell. Growth of lactic acid bacteria or yeasts in understerilized canned sauerkraut will yield carbon dioxide. Can corrosion may cause the liberation of hydrogen.

OLIVES

The first olive trees grown in California are said to have originated from seeds planted at the Mission San Diego in 1769. According to Lelong,[63] the seeds were brought from San Blas, Mexico, by Don Joseph de Galvez, while on an expedition to rediscover the port of Monterey. The seedling trees thus obtained are thought to be the source of the present Mission variety of olive (*Olea europaea*)

Although olives were utilized for oil production in the California Missions as early as 1780, they were of little commercia importance until about 1900. The first commercial production o olive oil outside of the missions did not occur until 1871. Prio to 1890, little attention was given to the utilization of olives fo commercial pickling, although pickling had been practiced fo some time on the farms and in the homes.

Until the development of the California ripe olive, olive oi was the chief product of the industry. The first attempts at commercial production of California ripe olives were made betwee 1890 and 1895. By 1900, there were several commercial producer of ripe olives. The development of California ripe olives was successful and, with modern technology, achieved worldwide prestig During the same period, attempts were also made to produce commercial quantities of Spanish-type, fermented green olives.[93] I contrast, however, the pickling of green olives has been of no grea importance in California until comparatively recently, despite th fact that, according to Dunn,[20] the Spanish Queen olive, or Sevillan variety, was introduced into the state some time before 1866.

That the canned ripe olive originally developed and even no produced only in California is the most important product of th California industry has already been shown in Table 40.

Varieties and Harvesting of Olives

COMMERCIAL VARIETIES

There are five main varieties of olives grown in bearing acreag in California. The Mission variety (of California origin), whic constitutes a little over half of the total bearing acreage of olives i the state, is followed in commercial importance by the Manzanillo, Sevillano, Ascolano, and Barouni varieties in the order named. The Manzanillo and Sevillano varieties were imported from Spain. The Ascolano variety originated in Italy. The Barouni variety was obtained from Tunisia. Each of these varieties is quite distinctive in size, shape, and general utility.

The different species vary markedly in size of fruits. The Sevillano olives are the largest, followed in order by the Ascolano, Barouni, Manzanillo and Mission varieties. The Manzanillo variety most nearly approaches the requirements of an all-purpose olive, since it may be used for the production of California ripe olives, green fermented olives, or olive oil. The other varieties have characteristics which limit their utility in one way or another. The Sevillano or Queen olive is desirable for production of the largest sizes of ripe or green fermented olives, but bears too little oil to be of great value for the production of olive oil. The Mission variety is best utilized for the production of ripe olives or oil and is not well suited for making green fermented olives. The Ascolano variety is used primarily for the production of canned ripe olives because its oil content is too low for economical recovery and its color is too light a green for good green fermented olives. The Barouni olive may be used to a certain extent for the production of green fermented olives but is not acceptable for the preparation of good quality California ripe olives. Consequently, a significant amount of the Barouni crop is shipped fresh from California to the eastern United States where the fruit is purchased for home pickling.

All of these varieties have been used for the production of other olive products, including Sicilian-type green olives and brined or dry salt-cured Greek-type olives, products not well known to the general public.

HARVESTING

Most of the olives destined for ripe or green pickling are harvested between October 1 and November 25, the time depending on the variety of fruit, the locality, the growing season, and other factors. Olives which are too green do not process well. Those picked near the end of the season often become undesirably softened or discolored and are prone to spoil more easily during the early stages of the pickling process.

Unfortunately, fresh olives are quite easily bruised. Care must be exercised in picking the fruit as well as in transportation an handling during preparation for pickling. To avoid unnecessar bruising, the olives are picked into padded buckets. The bucket of fruit are then emptied into boxes known as fruit lugs. Thes boxes are fitted with cleats to protect the olives against bruisin and crushing when they are stacked and transported. The lug vary in size, but the majority contains about 36 lb of olive (Figure 28).

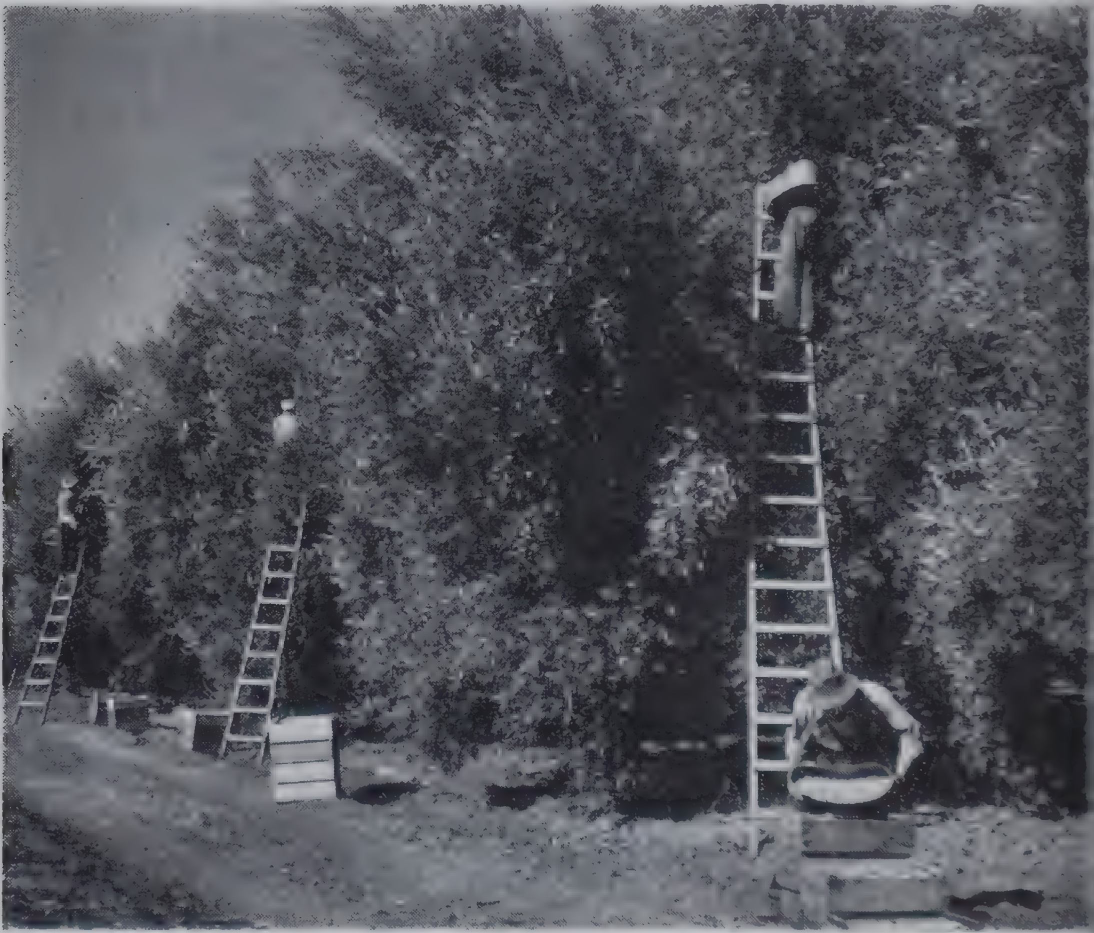


FIGURE 28. *Harvesting Olives*

Because the canned ripe olive is the chief product of the Cal fornia olive industry, harvesting practices naturally favor the s lection of fruit most desirable for production of this commodit However, high labor cost prevents the use of as much hand lab in California as is customarily used in other olive-growing are of the world.[10,12,48] When the majority of the fruit in an orchar has reached the desired maturity, the harvest is started and co tinued until all the olives destined for pickling have been gathered. The fruit in the top of the tree generally is more mature and is picked first. The industry is fully cognizant of the value of careful harvesting and selection of the fruit for maturity, but with prevailing economic conditions, it is impossible to maintain the desired high standards for picking.

Mature (ripe) olives do not have the final dark purple to jet-black color at the time of harvest for pickling, but are at the color-turning stage, from green to straw yellow or, at most, light cherry red. More highly colored fruit is overripe, deteriorates rapidly during the pickling processes, and consequently is used for the production of oil or preparation of such specialties as dry salt-cured or brine-cured Greek-type olives. Unfortunately, there are no chemical or physical methods for determining maturity and it is left to the field men to estimate maturity by empirical means.

California ripe olives (brown to black in color after canning) are made from fruit ranging in color from green to cherry red at the time of picking. The less well-known California green-ripe or "home-cured" olives (yellowish-green to light brown, frequently mottled color after canning) are made from olives having a green to not more than cherry-red color. California green olives are produced from fruit ranging from green to straw yellow.

The harvested fruit is transported to the pickling plants by truck or other motorized equipment. Use of cleated lug boxes (Figure 28) allows the fruit to be hauled without undue bruising and crushing.

Preparation and Processing

The only olives produced in significant commercial quantity without lactic acid fermentation are small quantities of California ripe olives pickled directly from the trees; California green-ripe olives which of necessity must be prepared immediately after harvest; and salt-cured Greek-type olives which are dehydrated and preserved in large quantities of dry salt. Since these olive products are not allowed to ferment, they will not be discussed here. For descriptions of their preparation the publications of Cruess[17] and Vaughn[87,88] should be consulted. All other types of olives undergo at least some lactic acid fermentation in salt brines.

Sorting and Grading

On arrival at the factories, the olives generally are carefully graded for defects, color, and size. All defective and damaged fruit is removed by hand as the olives are passed along sorting tables on endless belts. Color grading is accomplished in the same manner and it is customary to sort the olives for color into three main grades, green, cherry-red, and black fruit. The green fruit is then used for green fermented or ripe olives. The cherry-red fruit is used for ripe pickling and the black (overripe) fruit is used for oil or other by-products.

Whereas sorting and grading for defects and color is largely a hand operation, size grading is entirely mechanical. Size-grading machines consisting of diverging steel cables are almost exclusively used (Figure 29). The olives are graded into sizes according to the number of olives per pound, as shown in Table 43.

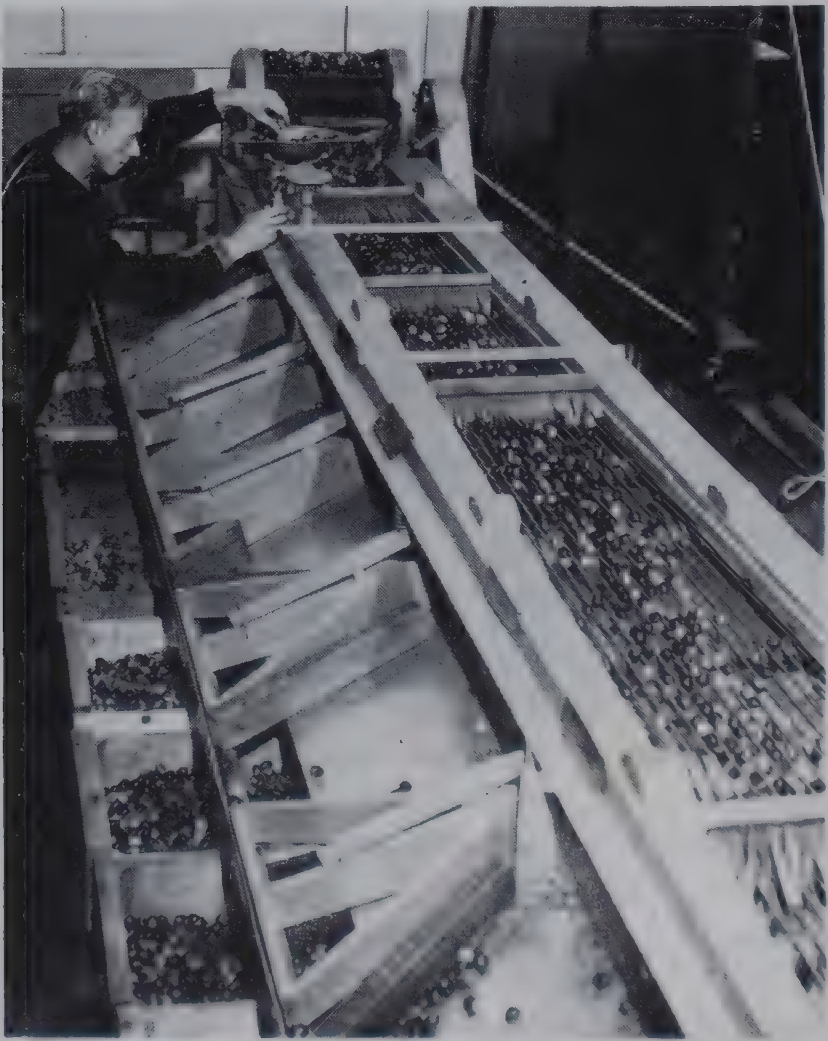


FIGURE 29. *Size Grading of Olives* (Courtesy—Pacific Olive Co.

Three blends of olive sizes—family, king, and royal—have been standardized recently in an attempt to reduce the number of sizes of olives now canned.[10a] The family blend, a mixture of the medium, large, and extra-large sizes, ranges between 91 and 105 olives per pound. The king size, a blend of the giant, jumbo, and smaller colossal olives, ranges between 45 and 53 olives per pound. The royal size is a blend of the largest olives not to exceed 34 olives per pound. If these new sizes are accepted in retail channels the number of sizes eventually will be reduced from nine to five and will include standard (a very competitive size), family, mammoth (a size that cannot be blended in all varieties), king and royal.

Rapid handling of the fresh fruit is necessary to avoid "sweating" which may predispose the olives to softening and discoloration. Therefore, when fresh fruit is being received in great abundance at the height of the harvest, it is sometimes necessary to forego size grading and to handle the fruit "orchard-run" until it is pickled and ready for final packaging.

TABLE 43. SIZE GRADES OF CALIFORNIA OLIVES[a]

California standard size grades	Count per pound (average)	Count per kilogram (average)
Super-colossal	32[b]	60-70
Colossal	36-40	80-90
Jumbo	46-50	100-110
Giant	53-60	120-130
Mammoth	70	140-160
Extra-large	82	170-190
Large	98	200-230
Medium	113	240-260
Small, select, or standard	135	280-310

[a] Based on the California Ripe Olive Standardization Act as amended in 1933 and 1935.

[b] Maximum number of olives allowed.

CALIFORNIA RIPE OLIVES

As already stressed, the California canned ripe olive may be prepared as soon as the fruit is harvested. However, most of the factories do not have enough pickling vats to handle all of the olives at once. Therefore, a large majority of the fruit destined for production of canned ripe olives is held in salt brine (holding solution) for some time until the pickling vats are available.

The strength of the brine used for holding olives prior to ripe pickling varies according to the type of fruit. The large varieties

(Sevillano and Ascolano) are very susceptible to salt shrivel. Consequently, it is customary to use a brine of 2.5 to 5% salt content (calculated as grams NaCl per 100 ml of brine) to cover the large varieties. Since the smaller Manzanillo and Mission varieties are comparatively immune to salt shrivel, they are generally placed in holding brines which contain 5 to about 10% salt.

The salt content of the original brine decreases because the olives take up salt from the brine by osmosis. It is necessary to in-

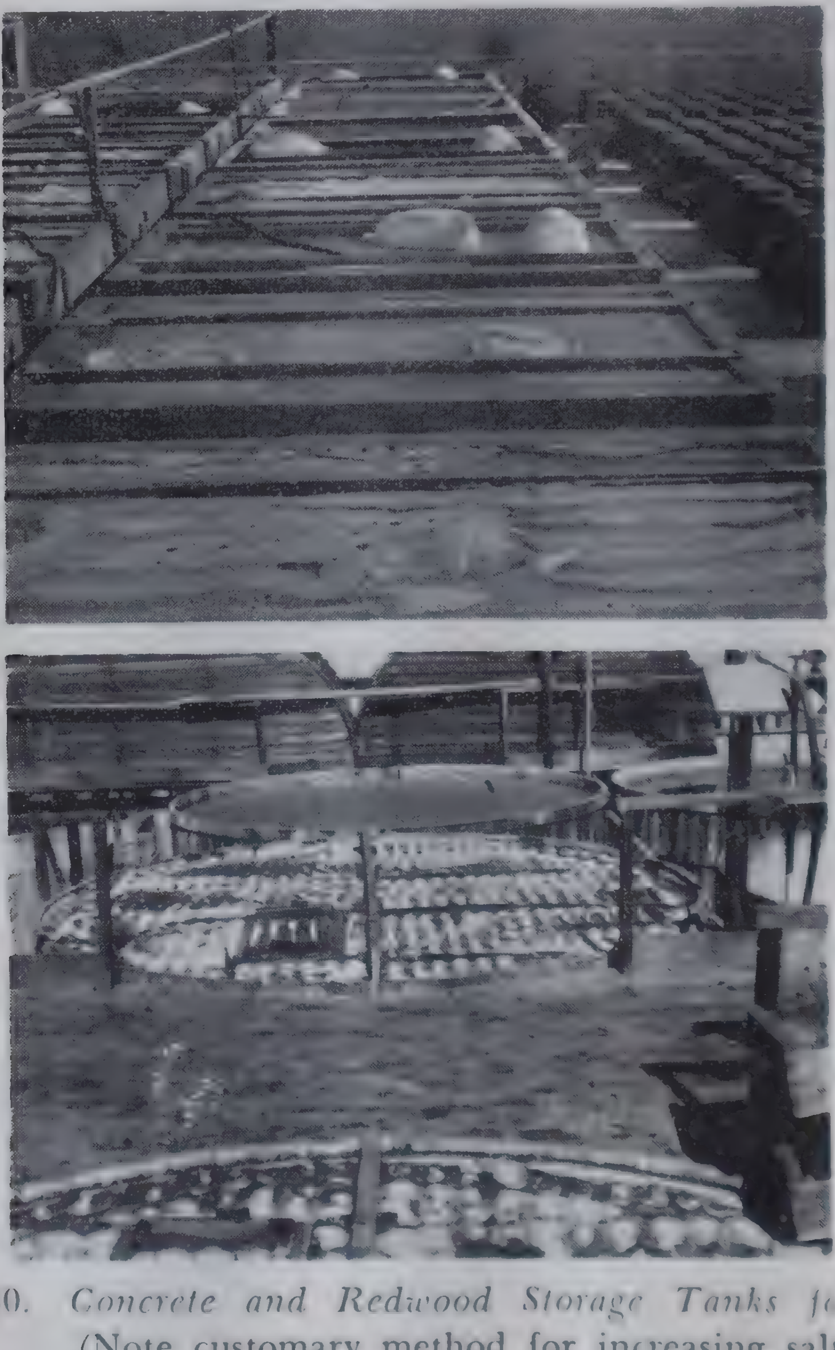


FIGURE 30. *Concrete and Redwood Storage Tanks for Olive*
(Note customary method for increasing salt conten
by dissolving sacks of salt in surface layer of brine

crease gradually the salt content of the holding solutions. Experience has shown that olives stored in brine keep best in the presence of 7 to 8 or even 10% sodium chloride.

It is customary to store the olives in the pickling vats or regular storage tanks, both of which may be constructed of reinforced concrete or redwood staves (Figure 30). The filled vats or tanks are closed with slatted covers which keep the olives submerged. If the brining is properly controlled, the olives cured in holding solutions can be stored safely for 6 to 8 months, or even longer.

PROCESSING OF RIPE OLIVES

The characteristic dark-brown to black color of the California canned ripe olive is obtained by oxidation of unknown constituents in the fruit by treatment with dilute lye (sodium hydroxide) solutions alternated with exposure to air or to highly aerated water. After lye treatment and oxidation, which fix the color of the olives, the excess caustic is removed by leaching with water. Finally, the olives are soaked in dilute salt brine (2.5 to 3.0% sodium chloride) to stabilize the salt content of the fruit just prior to canning.

The number of lye applications varies from 3 to 6 or 8. The strength of the caustic solution is between 0.5 and 2.0% sodium hydroxide, depending on the variety of fruit, the views and experience of the processor, and other factors. The first lye is allowed to penetrate just through the skin of the majority of the olives in the pickling vat. The first lye is thought to intensify the color change caused by the oxidation. Subsequent applications of sodium hydroxide also aid color fixation and hydrolyze the bitter glucoside *oleuropein,*[8,18] which, if not destroyed, leaves the olives so bitter as to be almost inedible. The last lye solution must penetrate the pit to destroy the last traces of the glucoside.

Each application of sodium hydroxide is followed by a period of oxidation. During this time, the olives must be stirred frequently to prevent uneven development of color when contact surfaces of the fruit are not exposed to the air. The unexposed surfaces do not darken to the same intensity, thus giving a mottled appearance to the fruit. For stirring, the olives are covered with water and the liquid and olives are agitated for several minutes with large wooden paddles or rods through which air is blown. Alternatively, oxidation is accomplished by covering the olives with water which then is continuously aerated by blowing air through a distributing device placed in the bottom of the pickling vats.

Excess lye is removed by leaching the olives with water unti all traces of the sodium hydroxide have disappeared. The watei is changed frequently and stirred often to facilitate leaching. Acidulated water is sometimes used to neutralize a portion of the sodiun hydroxide. Removal of the sodium hydroxide from the fruit as well as progress of its penetration, generally is followed b placing phenolphthalein on the cut surfaces of the olives.

After removal of the sodium hydroxide, the olives are stabilize with dilute salt brines prior to canning. Three changes of brine ar normally used for stabilization. The first contains 2.0, the secon 2.5, and the third 3.0% sodium chloride. This stabilization insure a uniform concentration of salt in the brine and in the olives afte canning.

When the olives have been subjected to a final sorting an grading, they are canned in a salt brine of about 3.0% sodiu chloride content. All canning is under the supervision of the Cal fornia State Department of Health. It is required that all canne olives are heat processed at 240°F for 1 hour.

Brined Greek-Type Olives

Brined or wet-process Greek-type olives are usually made fro firm yet highly colored purple to jet-black fruit. The color m also be intensified by lye treatment and oxidation as describe before. However, since this product is bitter, only one or at mo two lye applications can be used.

The olives are sorted and graded for size, placed in 16, 3 or 50-gal barrels, and covered with brine containing 7 to 10 sodium chloride. The salt content of the brine is then increase until it is about 15% or more. The olives cure out slowly, lo some of their natural bitterness, and take on a purple color. Litt is known about the microorganisms involved in the fermentatio It is obvious that the high salt content of the brine suppresses ac formation. Since yeasts abound, it is assumed that they utilize t fermentable carbohydrates present in the olives.

Only small quantities of these olives are produced. Most su able olives are harvested before they have reached the extre aturity required for making brined olives of this type. Furthernore, the natural bitterness and saltiness of the brined Greek-type live do not appeal to the normal American taste.

REEN FERMENTED OLIVES

There are two characteristic kinds of green fermented olives roduced in California: Spanish- and Sicilian-type olives. Formerly, icilian olives were the principal fermented product in California. ince about 1938, however, Spanish-type olives have been produced n increasing amounts.

PANISH-TYPE OLIVES

Fresh olives of green to straw-yellow color are treated with ye to destroy most of the bitter glucoside. The lye solutions which ontain 1.25 to 2.0% sodium hydroxide are allowed to penetrate o about three-fourths or more of the way to the pits. A small uantity of untreated flesh is allowed to remain, for a slight bitteress is essential to give the fermented fruit part of its flavor.

After lye treatment, the residual sodium hydroxide is removed rom the olives by leaching with water. During the leaching process, are is taken to avoid undue exposure of the olives to air or to erated water; otherwise, they darken undesirably. After removing he excess lye, the olives are placed in 50-gal barrels, covered with alt brine, rolled into the fermentation yard, and allowed to undergo lactic acid fermentation (Figure 31). Experimental bulk fermenations of green olives have been made in redwood tanks which old about 15,000 lb of olives. These studies indicate that there is o significant gross chemical or bacteriological difference between he barrel and the bulk processes. The bulk fermentation, if used nder the direct supervision of trained personnel, is economical of abor and time and insures a greater uniformity of product than oes the conventional barrel process.[4]

The concentration of salt in the brines varies widely. For the evillano fruit, it is customary to use a brine containing not over % salt at the time of brining. With Manzanillo and Barouni lives, it is customary to use a brine of 7 to 8% salt. The fermentaion takes place, as a rule, in the presence of about 4 to 7% salt.

The fermentation, which generally requires 6 months or longer, epending on the amount of laboratory control, results in the

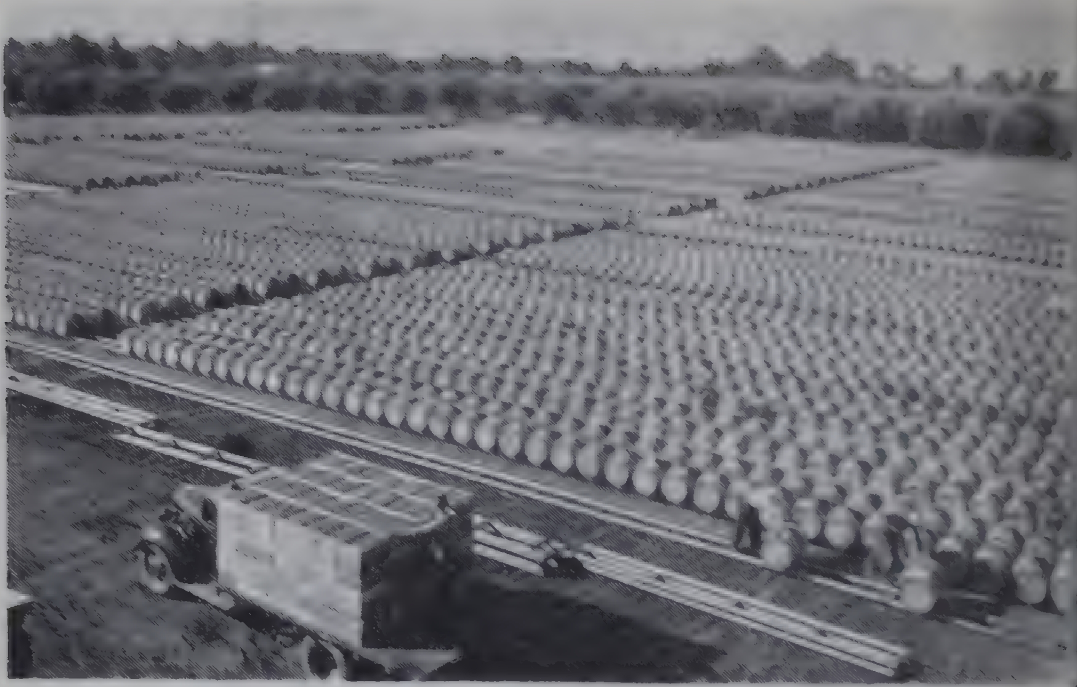


FIGURE 31. *Barrels of Olives in a Fermentation Yard* (Courte
Lindsay Ripe Olive Co.)

production of 0.7 to over 1.0% total acid (calculated as grams la
acid per 100 ml of brine). After the fermentation, the olives
ready for packing in glass or for shipment to repacking centers.

SICILIAN-TYPE OLIVES

Sicilian-type olives are made from green to straw-colored
even light-pink-colored Sevillano fruit and are fermented witł
lye treatment to remove the bitter glucoside *oleuropein*. For
process, suitable olives, after grading and sorting, are immedia
packed in 32-gal paraffin-lined barrels. The barrels of olives
then rolled into the fermentation yard, placed in an upright
tion and filled with water or a weak salt brine until the entire
of the barrel is covered. Salt is then added until the concentra
in the brine has increased to 7 or 8% (calculated as grams NaCl
100 ml of brine). The olives undergo a lactic acid fermenta
similar to that observed in holding solution (storage) olives ex
that conditions in the barrels suppress the excessive populat
of yeasts generally found in storage brines.

Formerly, it was the practice to add spices when the o
were barreled. Now most of the Sicilian-type olives are ferme

nspiced brines and, after fermentation, look and taste almost tical with storage fruit.

Fermentation

The important, normal floral and chemical changes occurring he brines of fermenting olives have been found to be similar hose reported for the fermentation of sauerkraut by Pederson[71] for pickles by Etchells and Jones.[27,28] There are, however, ortant differences in the rates of fermentation. Olives, under conditions which exist in the California industry, do not ferment rly as rapidly as either cabbage or cucumbers.

Seasonal temperatures play a major role in the rate of fer- ntation of olives. Even during the earlier part of the season tober 1 to 15), the temperatures of the fermenting brines rage, Sicilian or Spanish-type), in most cases, do not increase ificantly above the temperatures of the water supplies with ch the brines were made. Temperatures averaging 68°F are mal for brines during this period. Later, the temperatures de- se markedly and, during the winter months, average between and 45°F.

The concentration of salt, availability of fermentable carbo- rates, and the presence or absence of desirable bacteria, as be shown, also affect the rate of fermentation. It may also be med that there are differences in naturally occurring bacterial wth factors and antibiotic substances in olives as compared with er cabbage or cucumbers.

MENTATION IN HOLDING SOLUTIONS

Although detailed studies have not been published, it is known n the work of the author and his students that olives held alt brines undergo a typical lactic acid fermentation caused by same types of bacteria as those found in the sauerkraut and umber fermentations.

The acidity developed is somewhat variable, but usually ranges ween 0.4 and 0.6% (total acidity calculated as grams lactic acid 100 ml of brine). The development of acidity is usually not pronounced in holding brines, although the sugar content he different varieties of olives is sufficient to allow it. The high concentration of some of the brines; an abundance of yeasts in the brines; and the low temperatures of the brines during the late fall and winter months all tend to prevent, suppress, or reduce the acidity.

The major varieties of olives differ appreciably in their fermentability in holding brines. The Ascolano and Sevillano varieties ferment very readily, whereas the Manzanillo and Mission varieties ferment slowly. The apparent varietal differences are correlated with the content of fermentable sugars. The Sevillano and Ascolano varieties contain the most, the Manzanillo and Mission varieties the least sugar. Their order in oleuropein content (which may eventually be shown to suppress the activity of the lactic acid bacteria) is exactly reversed. It has been stressed previously that the Sevillano and Ascolano varieties are quite sensitive to salt shrivel and must be stored in brines containing less salt than the other varieties. It is known that the rate of fermentation is decreased as the salt content of the brine is increased. It is obvious, therefore, that the Sevillano and Ascolano varieties should ferment more rapidly under commercial conditions.

SICILIAN-TYPE FERMENTATION

The gross chemical and bacteriological changes that occur i the brines of Sicilian-type olives are similar in every respect to thos found in holding-brine fermentations. Normally, all of the Sicilia type olives are made from Sevillano fruit. The total acid productio in the Sicilian-type fermentation is somewhat higher than i storage brines. The fermentation takes place in barrels and consequently is more anaerobic (Figure 32). The barrels are store in the open where the surface brines are irradiated by the su These two factors combine to markedly reduce the large populations of acid-destroying yeasts normally present in holding brine

SPANISH-TYPE FERMENTATIONS

It is generally acknowledged that successful, large-scale production of Spanish-type green olives depends in large measure o a thorough knowledge of the changes which occur during preparation and fermentation of the fruit. Consequently, these change are better known than those which occur in other fermented olive

Although early attempts were made to produce Spanish-typ green fermented olives in California, there was no significant commercial production until comparatively recently. The first extensi

FIGURE 32. *Barrel of Fermenting Sicilian-Type Olives*

ommercial-scale experiments made to study this fermentation were eported by Cruess.[13] Since about 1935, there has been a gradual ncrease in the interest in production of this olive. The increased roduction is the result of conscientious efforts of the whole industry o adapt the fermentation process to conditions which prevail in alifornia. It is now recognized throughout the industry that the reen-olive fermentation must be rigorously controlled. There are everal reasons why control of the fermentation is mandatory if t is to be successful.

To insure the production of lactic acid which, together with alt, preserves the fermented olives, it is necessary to have a suffi- ient quantity of fermentable matter present in the fruit. Also, he brine and olives must have a concentration of salt that will not e harmful to any desirable lactic acid bacteria which may be resent. Finally, the brined olives must be held at a temperature hich will allow the desirable bacteria to dominate the fermentation.

Fresh olives of the varieties desirable for green pickling do, ccording to Nichols,[89] contain enough fermentable substances glucose, fructose, sucrose, and mannitol). However, as Cruess[13] as shown, up to 65% of the total fermentables may be lost by lye treatment and washing to destroy the bitter glucoside. Therefore, failure of the olives to ferment properly may result from lack of fermentable sugars.

According to data accumulated by the author and his students, lye treatment and use of too concentrated salt brines can deplete the population of desirable lactic acid bacteria to such an extent that they cannot compete with undesirable bacteria and yeasts even if sufficient sugar is present. Therefore, to insure fermentation of green olives, it is necessary to add supplementary sugar and to insure the presence of desirable lactic acid bacteria. However, unless the temperature is controlled by artificial means during the winter months, fermentation may cease, for it is well known that the lactic acid bacteria have minimum temperature ranges for growth and acid production. A more detailed discussion of some aspects of the control of the fermentation is given by Vaughn, Douglas, and Gililland.[89] It is desirable to control the fermentation until the pH of the brine has decreased to a value of 3.8 or less. Under such conditions, the total acidity calculated as lactic acid will be in the range of 0.7 to 1.0% (calculated as grams lactic acid per 100 ml of brine). It is possible, by proper manipulation, to complete the fermentation in 3 weeks to 1 month under commercial conditions. To accomplish this, 2 to 5 lb of supplementary sugar must be added to the brines; desirable lactic acid bacteria must be present in the brines; the salt concentration must be at 5 to 6% (calculated as grams NaCl per 100 ml of brine); and a temperature of at least 75°F must be maintained. *These control measures must be carefully integrated.* Inoculation with a starter will not accelerate acid production unless there is sufficient fermentable sugar present in the brines. It is unreasonable to expect appreciable acid production in the presence of 10% salt or in brines at 45°F, regardless of other favorable conditions.

Crystallized corn sugar (glucose) is commonly used as the source of supplementary sugar, although cane or beet sugar (sucrose) may also be used.

To insure desirable populations of lactic acid bacteria, pure culture starters of various strains of *Lactobacillus plantarum* may be used.[14] In the absence of suitable facilities for developing and maintaining pure-culture starters, it is common practice to use quantities of normal, actively fermenting brine. In many cases for reasons as yet imperfectly understood, use of some kind of starter is necessary.

Incubation of the olives is accomplished by storing the barrels in special incubating rooms where the temperature is maintained at 75° to 85°F. For obvious economic reasons, only a portion of the crop is incubated.

The sequence of bacteria observed in the fermentation of olives is essentially the same as that described in Table 42 for the fermentation of cucumbers in low-salt brines. However, because the olives ferment more slowly, the changes in microbial populations are more clear-cut and it is easier to delimit the different stages of fermentation.

As already indicated, the salt concentration of the brines has a marked effect on the total numbers and types of bacteria and other microorganisms found in them. Thus, the fermentations of the Manzanillo variety, which have been customarily conducted in the presence of 7 to 8% salt, have a different bacterial population than the Sevillano olives, which are fermented in the presence of from 3 to about 5% salt. Whereas both homofermentative and heterofermentative species of *Lactobacillus* have always been recovered from the brines of Sevillano olives, only the homofermentative species *Lactobacillus plantarum* has been recovered from Manzanillo brines containing 7 to 8% salt. *Leuconostoc mesenteroides*, being salt tolerant, is found in both fermentations.

This difference in bacterial populations is a manifestation of the differences in salt tolerance of the lactobacilli. When Manzanillo olives are fermented in the presence of 3 to 5% salt, the heterofermentative as well as the homofermentative lactobacilli are recovered. When Sevillano olives are fermented in brines containing 7 to 8% salt, only homofrmentative *Lactobacillus plantarum* is found. When either variety is fermented in the presence of 10% salt, it is difficult to recover any lactobacilli at all and there is no significant acid production in the brines even if supplementary sugar is constantly supplied for as long as a year.

Determination of the salt tolerance of pure cultures of lactic acid bacteria is difficult. However, these more recent observations concerning the salt tolerance of lactic acid bacteria from green-olive brines, together with those previously reported by Vaughn, Douglas, and Gililland[89] amply support the conclusions of Orla Jensen[70] that heterofermentative species of *Lactobacillus* are less resistant to salt than the species of *Leuconostoc* or homofermentative species of *Lactobacillus*.

Spoilage Problems

Bacteria, yeasts, and molds may cause deterioration of olives at any time between the harvest and the final packaging of the olive product. Bacteria generally are responsible for the most serious losses, although, at times, yeasts and molds may also be involved. Care in handling the freshly harvested olives and control of the course of biological, chemical, and physical events taking place during the primary stage of fermentation determine, in a large measure, the extent of deterioration of the fruit. However losses may result from general lack of adequate sanitation or from neglect of the fruit during any subsequent step in the process o preparing the various olive products.

The commonest and, consequently, best-known types of spoilage caused by the activity of microorganisms are softening, gassy deterioration, and butyric fermentation. Of these, gassy spoilage occurs most frequently and under the widest variety of conditions

GASSY, "FLOATER," OR "FISH-EYE" SPOILAGE

This deterioration (Figure 33) is characterized by the formation of blisters resulting from accumulation of gases which separate the skin from the flesh of the olives (not to be confused with blister caused by too concentrated or too warm lye solutions) or by the formation of fissures or gas pockets which may extend to the pits of the fruits. Since this type of spoilage has been extensively studied b Cruess and Guthier,[19] Alvarez,[1] Tracy,[85] and Vaughn and his students,[45,89,92] it is well established that the coliform bacteria are chiefly responsible for gassy spoilage.

The coliform species most frequently encountered are *Aerobacter aerogenes* and *A. cloacae*. *Escherichia freundii* and *E. intermedium* are also found, but no true *E. coli* cultures have been reported. Cultures of *Bacillus* (*Aerobacillus*) *polymyxa* and *B macerans* also cause gas-pocket formation in olives. Various species of the saccharolytic anaerobes of the genus *Clostridium* may also produce gas pockets in olives, but these butyric acid bacteria are more important for the malodorous fermentations they produce

The bacteria mentioned all produce hydrogen gas as an end product of the decomposition of glucose, fructose, and mannitol It is the contention of the author that only those bacteria which produce hydrogen gas are dangerous. Carbon dioxide is much more

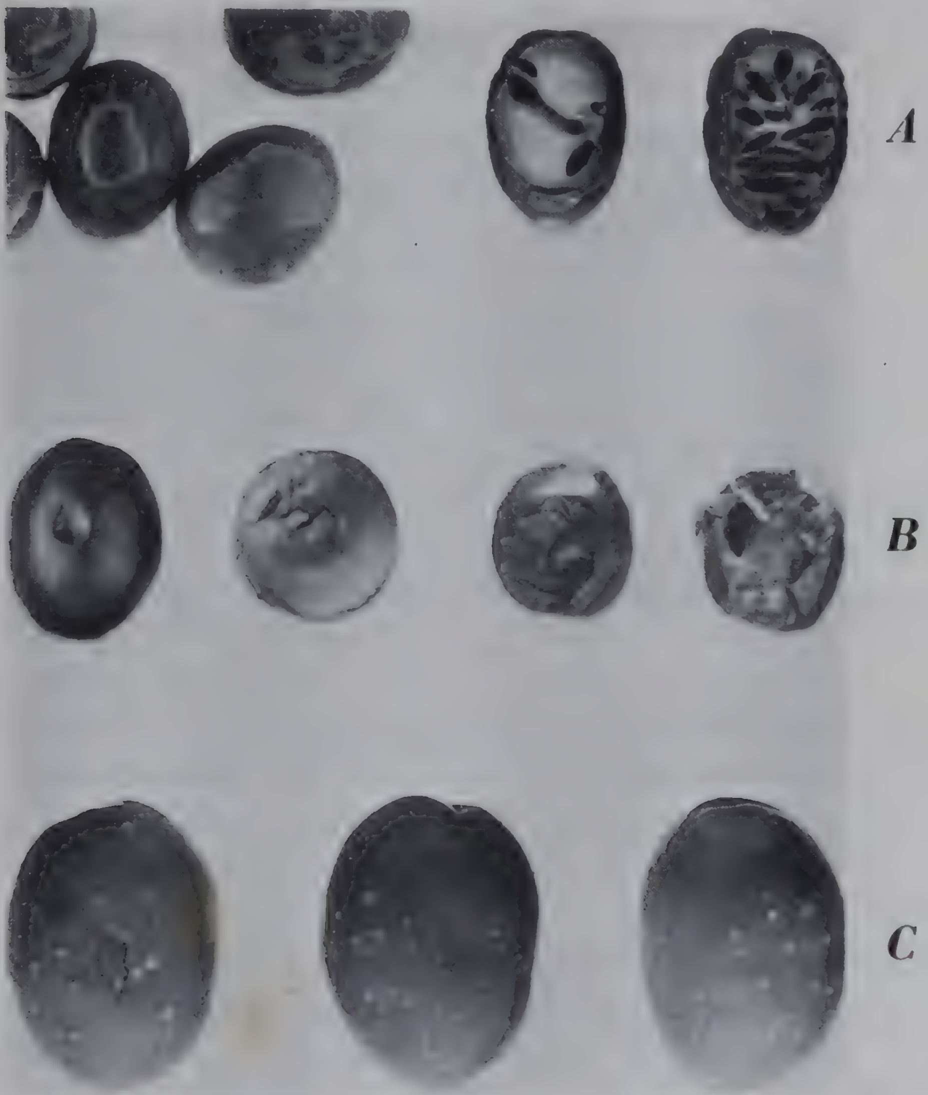


FIGURE 33. *Spoilage of Olives:* ***A*** *Manifestations of gas formation;* ***B*** *Softening, left to right, "nail-head," soft stem-end, mushiness and sloughing, respectively;* ***C*** *"Yeast spots"*

soluble than hydrogen. Yeasts and heterofermentative lactic acid bacteria (*Leuconostoc mesenteroides, Lactobacillus brevis,* and others) produce large quantities of carbon dioxide and abound in all olive fermentations; yet, pure-culture studies with them have failed to substantiate that either the yeasts or lactic acid bacteria cause gassy spoilage of olives.

Gas-pocket formation may occur in olives in storage brines, Sicilian-type fermentations, Spanish-type fermentations, or during the ripe pickling process, particularly in those olives pickled without preliminary storage in brine.

Conditions which favor the gaseous spoilage are to be found in an environment containing not more than 5% salt and having pH values of 4.8 to 8.5, a temperature of 75° to 85°F, and no significant population of lactic acid bacteria to compete with the coliform bacteria for an abundance of fermentable carbohydrates. It is obvious, therefore, that control measures to effectively eliminate serious losses resulting from gas-pocket formation should be preventive rather than curative. Coliform bacteria, particularly the *Aerobacter* types, are found in most olive brines during the primary stage of fermentation. If the fermentation proceeds in a normal manner, there is no significant production of gas pockets. If, however, this first stage is unduly prolonged, the coliform bacteria dominate the microbial populations in the brines and spoilage results. The prevalence of the coliform bacteria in nature makes it impossible to eliminate them completely. However, much can be done to effectively reduce the coliform populations by control of contaminated plant water supplies, thus preventing the plant from continuous inoculation with coliform bacteria. In commercial practice, control of the plant water supply, coupled with maintenance of a general high level of sanitation, will reduce gaseous spoilage to a minimum, if the usual attention is given to the olive fermentations themselves.

Nevertheless, in cases of acute spoilage where there is potential danger of complete loss of the fruit, speedy curative measures are dictated. These include the use of strongly acidified brines or pasteurization. Heating softens fresh or partially fermented olives and, therefore, speedy rebrining with acidified brine is recommended to check spoilage in such fruit. Pasteurization may be used particularly well in the ripe pickling of olives. However, commercial pasteurization will not kill the spores of the *Bacillus polymyxa-macerans* group under all conditions.

There is an extremely salt-tolerant form of *Aerobacter aerogenes*, which was recently described by Foda and Vaughn.[45] This salt-tolerant form will grow and produce gas in the presence of 14% salt (calculated as grams NaCl per 100 ml of brine). Obviously, salt alone will have no effect on the control of this particular type of coliform bacterium, which often occurs in olive brines.

MALODOROUS FERMENTATIONS

The butyric acid bacteria have been associated with the deterioration of olives since Hayne and Colby,[50] in 1895, first recorded their spoilage by the "butyric ferment." This abnormal fermentation is characterized by its butyric acid or rancid odor. In the early stages of the fermentation, the odor is distinctly that of butyric acid or rancid butter; but, as the spoilage progresses, the odor becomes increasingly obnoxious and finally results in a very malodorous, fecal stench.

Pure cultures of anaerobic, spore-forming butyric acid bacteria were first isolated from a large number of samples of spoiled olives by Gililland and Vaughn.[47] All of the cultures studied were sugar-decomposing types not capable of digesting protein. Most of the cultures were closely related to or identical with *Clostridium butyricum*. All of the isolates caused malodorous fermentation of olives if the chemical and physical conditions, such as salt concentration, pH, and sugar content in the brines, were favorable for their growth.

Butyric acid spoilage is less widespread than the abnormal gas-pocket fermentation commonly caused by the coliform bacteria. Nevertheless, losses may be extensive, because olives affected by butyric fermentation cannot be salvaged. Control, therefore, must be designed to prevent the activity of the butyric acid bacteria rather than to check the fermentation once it is started. Scrupulous sanitation and rigid control of the primary phase of the fermentation are the only feasible methods for preventing the butyric acid fermentation. If the fermentation has been carefully controlled until acid production has decreased the pH value of the brines to a value of 3.8 to 4.0, there is little danger that the butyric acid bacteria will spoil the olives.

"Zapatera" spoilage is another malodorous fermentation which occurs in holding brines, Sicilian-type fermentations, and Spanish-type fermentations of olives in California. This abnormality is characterized by the development of a very penetrating, unpleasant, "sagey" odor in olives undergoing fermentation. Under California conditions, zapatera spoilage, unlike the butyric fermentation, occurs when the desirable lactic acid fermentation is allowed to cease before the pH of the brine has decreased below 4.5. At the onset of deterioration, the pH of the affected brines increases and the titratable acidity decreases as spoilage progresses. Smyth,[83] who made a study of the bacteriology of the Spanish green olive, concluded that spoilage was due "to one or more of a group of spore-forming, proteolytic, facultative rods normally present in the soils of Andalusia."

The author has isolated large numbers of viable spores o *Clostridium butyricum* from the brines of certified samples of imported Spanish green olives affected with zapatera. Attempts to isolate other bacteria which produce evil-smelling compounds in olives have been unsuccessful. It is known that lactic and acetic acids disappear during development of the spoilage and are replaced by propionic, butyric, valeric, caproic and caprylic acids.[19a] Although causative organisms have not been proven, the author and his students have isolated a number of *Cl. butyricum* and related cultures that ferment lactic acid if acetic acid is also present.

Control of zapatera spoilage, to be successful, must provide the same measures as have been used to prevent the butyric acid fermentation.

SOFTENING

The softening of olives manifests itself in one of three characteristic changes in the appearance of the fruit. These changes are known in the industry by the very descriptive terms "soft stem end," "nail head" and "sloughing," respectively. Examples of these three types of softening are shown in Figure 33. These types of softening have been observed in all kinds of pickled olives. The changes are so characteristic and the common names are so indicative of the types of change that further description is superfluous.

Histological studies made by the author and his students have shown that softening of olive tissue results from destruction of the pectic substances which hold the individual cells together and give firmness and shape to the fruit.[69a] It was also reported that strains o *Aerobacillus* (the *Bacillus polymyxa-macerans* group of bacteria capable of elaborating pectolytic enzymes could cause softening o olives. Cruess[16] used a commercial pectolytic-enzyme preparation t demonstrate softening of olives after having observed softening o laboratory samples of olives caused by an unidentified species o *Pencillium*. It is known from the literature that other bacteria and fungi also elaborate pectolytic enzymes. However, the form and texture of fresh olives may also be altered by use of too concentrated sodium hydroxide solutions, by frosting, or by heating. Thu it is not always easy to determine whether the softening is o icrobial origin or whether it results from chemical or physical istreatment of the fruit.

Detection of softening resulting from microbial activity has een somewhat simplified recently. Baier and Manchester[3] have eveloped a pectate-gel medium which may be used for the direct solation of microorganisms which elaborate pectolytic enzymes. ell, Etchells, and Jones[5] have also devised a qualitative test for he enzyme polygalacturonase in cucumber brines. The author has sed the Baier-Manchester gel medium for the isolation of aerobic ectolytic microorganisms (strains of *Bacillus megatherium, Bacil-us macerans, Penicilli,* and *Aspergilli*) from olive brines. It is oped that further applications of the polygalacturonase-enzyme est will be made so that softening may be forecast in time to pre-ent severe losses in olives.

Although practical control at present is limited by lack of nowledge, softening is curtailed or eliminated by a normal lactic cid fermentation. Other preventive measures include careful andling of the freshly harvested olives to prevent "sweating." torage of the fresh fruit for more than 3 days before brining or lye reatment greatly increases the amount of possible softening. It is lso mandatory to maintain a high level of plant sanitation.

EAST SPOTS

One of the very common defects associated with fermented lives in California is the abnormality known throughout the in-dustry as "yeast spots." This defect is characterized by the develop-ment of raised white spots or pimples which form between the pidermis and the underlying flesh of the olive (Figure 33). Al-hough commonly called "yeast spots," most, if not all of the imples contain lactobacilli.[80a] These bacteria have been isolated nd studied, and they have been identified as *Lactobacillus plant-rum* and *Lactobacillus brevis* or other heterofermentative species losely related to *L. brevis.*

This defect is also found in green olives certified to have been mported from Spain and has been observed in cucumber pickles nd pickled green tomatoes. Evidently, any factor which influences he entrance of the lactobacilli through the pores (stomata) or ther breaks in the skin of the fruit may contribute to the develop-ment of the colonies of bacteria. Since such olives are considered unsightly, some loss of value of the pickled fruit is experience although the olives are perfectly normal and healthful in othe respects. No control measures are known.

REFERMENTATION

Unsightly sediments and gas formation frequently occur i glass-packed fermented green olives.[68,89] The principal cause fo this refermentation is a reservoir of fermentable sugars left in th olives or unwitting addition of fermentable substances contained i various spices and flavoring constituents used when the fruit packed in glass. If such substances are present, refermentatio with gas formation and development of turbidity and a heav sediment in the brine, appears quickly unless control measures ar used.

Most refermentation is caused by lactic acid bacteria. Howeve if the containers are not completely filled with brine or vacuum sealed to exclude air, yeasts may form much of the sediment an both yeasts and molds form a pellicle in the head space betwee the brine and the jar cap.

To control refermentation effectively, it must be carefully d termined that most or all of the fermentable matter in the olive brine, and other constituents has been decomposed. It is als desirable to wash the olives to remove lactic acid bacteria an yeasts adhering to the surface of the fruit. The brines should b made with high-quality salt and edible lactic acid. It is also nece sary to exclude as much air as possible either by complete fillin of the jar with brine or by use of vacuum-type jar closures. Pasteur zation may also be used to destroy yeasts and bacteria which migh otherwise cause refermentation. The use of any chemical preserv tive per se is not practiced in the California industry.

OTHER ABNORMALITIES

Heavy yeast and mold growths on the surface of brines coverin olives, whether in barrels or storage tanks, may also impart of flavors and aromas to the fruit. Aside from the off-flavors an aromas developed through such neglect, it is known that the yeas and molds rapidly destroy the lactic acid contained in the brine Loss of the lactic acid by oxidation in this manner makes the olive susceptible to other spoilage. Control of yeast and mold growth best accomplished by routine rebrining of the barrels with fres rine during active fermentation and keeping them tightly closed uring storage to eliminate air. Storage brines are easily kept free f film-forming fungi if the tanks are exposed to sunlight, but resent a problem if the tanks are roofed over. With recently eveloped pumping techniques for handling storage olives, howver, closed tanks could be used, thus eliminating the need for ultraiolet lamps, layers of oil, or other control measures.

Purely chemical deterioration of olives is caused by the same ontaminating metals (copper and iron) as have already been disussed with cucumber pickles. The problems are nearly identical nd the same preventive measures are required for both products.

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CHAPTER 12

SELECTION AND MAINTENANCE OF CULTURES

Elizabeth F. McCoy

It has been said that the working capital of a bacteriologist is his set of stock cultures. Certainly, they are valuable and he must handle them so as to preserve that value and to profit by it in their fermentation use. In fact, the culture is the first of the limiting factors in a successful commercial fermentation.

SELECTION OF CULTURES

The original selection of suitable microbial cultures for commercial use is of considerable importance. In every group of fermentation organisms, there is a variety of strains producing the desired product, and it is important to select those (a) capable of highest yield under the commercial conditions to be used and (b) stable enough to give that yield consistently. Often, stability is more important, because in the plant, there is greater need for a vigorous, dependable starter culture than for one giving the highest, but erratic yields in laboratory tests. It may even be necessary to settle for the use of a good, but not high-yielding strain for this reason.

It is difficult to generalize on the original selection of the cultures. There are some, like the lactobacilli, which can be freshly isolated relatively easily and such fresh isolates from nature are likely to be superior to old laboratory cultures. For example, the following procedure for isolation of *Lactobacillus delbruckii* (or the recently recognized spore-forming lactic organisms, such as *Bacillus dextrolacticus*) is likely to yield a high acid-producing strain.

It is well to start with several potential source materials, such as grains, malt sprouts, and soil, because, while *L. delbruckii* is widespread, one cannot assume it present in every sample. Tubes of 5% glucose-yeast water or malt broth (deep broth to favor the microaerophilic lactobacilli should be inoculated). Incubation should be carried out at 45° to 55°C for 24 to 36 hours, at which time high acidity should have developed with resultant killing of the less acid-tolerant lactobacilli. Transfer should be made one or more times to 10% glucose broth containing excess calcium carbonate added as a sterile slurry. Growth under these conditions, at high temperature in high-sugar medium, results in further selection in favor of *L. delbruckii.* One may even use 12 or 15% glucose broth at this stage of culture provided that rapid transfers, e.g., at 12 to 24 hours, are made so as not to lose the vigorous *L. delbruckii* enrichment culture. When *L. delbruckii* is in the desired state of dominance as shown by microscopic evidence, the culture may be plated and a pure culture picked. Incubation of plates under anaerobic conditions is desirable for the lactobacillus in question.

In our experience, this procedure is very suitable for the isolation of *Lactobacillus delbruckii* from the various lactobacilli probably present in the original source inoculum; if any interference is encountered, it is generally due to the spore-forming lactic organisms, *Bacillus dextrolacticus,* and related forms which will develop under the same enrichment conditions. Such a lactic culture, if isolated, may be useful, since it is a strong lactic fermenter. But, if one wishes to avoid the spore former, one may detect it (and not use the enrichment for plating) by microscopic examination. The spore-forming organism in vegetative state is a slender Gram-negative rod in contrast to the Gram-positive, but variable rods which are the lactobacilli. In addition, the spore formers are strongly catalase positive and a catalase test with hydrogen peroxide on the enrichment culture will be positive, whereas it should be negative for a lactobacillius enrichment.

In other cases, the finding of a desirable strain for a commercial ermentation is much more difficult, but can still be done with certainty provided the microbiologist sets up proper selective enrichments and is persistent until he isolates a desirable strain for his purpose. Let us consider, for example, the problem presented by the butyl-butyric group. They are members of the genus *Clostridium*, which is a large and heterogeneous group containing pathogens and putrefactive members as well as the desired butyl-butyric fermentative types. Enrichment of the butyl-butyric anaerobes is relatively simple, if the primary cultures are started in starch or sugar media to select in favor of the saccharolytic anaerobes. It is important also to eliminate non-spore-forming contaminants at the outset, because lactobacilli, if present, would seriously interfere with the desired enrichment of butyl anaerobes. They may be eliminated by heating the inoculum for the original culture, e.g., by a treatment for 1 to 5 minutes at 100°C. Even at best, it is necessary to start many enrichments to get a few desirable ones. If one wishes to isolate the butyl types, one must expect them to be present in only 25 to 30% of the original source materials; butyrics, or at least low-producing butyl types, are contained in many more natural materials and it is thus necessary to distinguish the enrichments in which the butyl types appear in sufficient numbers to be successfully isolated. This may be done, to some extent, by noting those tubes in which the odors of butyl alcohol and acetone are pronounced and plating from such rather than from tubes with cheesy, butyric odors. One might think that to get the butyl organisms in dominant numbers, subculture in series would be attempted, but this is not done. In fact, the butyl types do not compete well in serial transfer and may even be lost by such a procedure before plating. The better plan is to allow sporulation of the primary or secondary culture for 5 to 7 days and then to plate from spores for isolation. Even so, it is necessary to persist until the desired type of butyl anaerobe is obtained, e.g., types for corn versus molasses (based on good utilization of starch versus high sugar); butyl-acetone versus butyl-isopropyl alcohol; and especially the highest ratio of butyl alcohol to other solvents. The chances of getting an outstanding culture of the desired type are certainly small, but it is possible to obtain them within reasonable time. It appears that the butyl patent situation reflects the culture situation. When an outstanding culture is found, it is likely to be characterized as a new species and its use in a fermentation process made the basis of a new butyl patent. Such a situation is justifiable only in view of the rarity of outstanding butyl cultures and by the fact that diversity of species does exist in the group (in contrast to the situation in the *Lactobacillus* group in which the single species, *L. delbruckii,* in the broad sense, covers the type of industrial interest).

Lastly, there are industrial starter cultures, which represent purely chance findings. The new user would be well advised to obtain his starter culture from someone who has it, rather than to attempt isolation. The penicillin cultures are good examples. From the first *Penicillium notatum* of Fleming to the currently used strains of *Penicillium chrysogenum,* there has been a long program of isolation and selection of better strains and, interestingly, in the latter period of development, the greatest progress was made by selecting substrains within the parent cultures. This was done by a combination of plating and selection of colonies, which by chance were better than their parent (Raper, Alexander, and Coghill,[22] and Raper and Fennell[23]), by induced variation (mutation) by X-ray (Demerec[5]), and by ultraviolet radiations (Backus, Stauffer, and Johnson[2]). The development of the present strains represents an immense amount of research effort and time by government, university, and industrial research workers before efficient starter cultures were ready for plant use. In such cases, it is wise to make arrangements for use of the best available culture rather than to attempt new isolations.

MAINTENANCE OF CULTURES

There is no single best method of maintenance of stock cultures. The two objectives are maintenance of viability and preservation of the original desirable characteristics of the cultures. Obviously the first must be achieved or all is lost, but it is not always realized that the second objective does not necessarily accompany the first. Degeneration of the old stock cultures is very common and is well recognized by specialists.

There is one principle that can be highly recommended, i.e. the choice of culture medium and/or conditions of storage that will allow the least number of culture transfers, so as to avoid slow degeneration and variation (mutational) loss of the desirable char cters of the cultures. Everyone who carries stock cultures is (or hould be) aware of this. As a result, there is very extensive literaure on stock-culture methods. Much of that literature does not pply to industrial stocks, because it deals with pathogens and the ffort to maintain virulence as well as viability. But some of the principles and claims can well be considered here.

Paraffin-Oil Method

The use of paraffin-oil seal, although an old technique, has recently been favorably reported on (Morton and Pulaski,[18] Hac,[12] Simmons,[25] and Gordon and Smith[10]). Actually, this is the method of Ungerman (Michael[17]) and consists merely of culture of the organisms on a suitable medium until they are well grown and then of layering with sterile mineral oil to a depth of perhaps 1 cm. The oil seal checks water evaporation, which is desirable, since water loss, with consequent increase in osmotic pressure and salt effects, is in part the cause of death in old cultures, as they approach air dryness. At the same time, the oil seal allows slow diffusion of oxygen into the culture, as is evident from slow growth of obligate aerobes under the seal. It also allows transfer by loop of culture material to a new culture medium without destroying the parent stock. From our own experience, such an open stock is very convenient and time saving.

In the report of Simmons,[25] the paraffin-oil method was recommended for a variety of pathogens, such as staphylococci, streptococci, and diphtheria bacteria, which survived 15 to 30 months in storage at room temperature in the dark. Of particular interest is his report that *Neisseria meningitidis* survived 12 months even at 37°C in the paraffined sealed stock; this result is apparently not a chance observation, since Hac[12] also reported paraffin-oil storage favorable for *Neisseria gonorrheae.* He even found it superior to lyophilizing *N. gonorrheae.* Another notoriously difficult culture to keep in stock is *Phytomonas sepedonica,* which Sherf[24] succeeded in keeping 18 months without transfer under oil seal. Perhaps because of such recent recommendations of the oil method, Gordon and Smith[10] report that tests are underway at the American Type Culture Collection with species of some forty-two genera of bacteria. In a preliminary report, they stated that tests were being made at 6-month intervals and that the preliminary indications were of success with: *Achromobacter, Actinomyces, Agrobacterium, Bacterium, Brucella, Caseococcus, Cellulomonas, Chromobacterium, Coccobacillus, Corynebacterium, Eberthella, Erysipelothrix, Escherichia, Flavobacterium, Gaffkya, Klebsiella, Kurthia, Listerella, Microbacterium, Micrococcus, Mycobacterium, Mycoplana, Neisseria, Pasteurella, Rhizobium, Sarcina, Serratia, Shigella, Spirillum,* and *Xanthomonas.* They added that "The method was unsatisfactory for some cultures of the following genera: *Aerobacter, Alcaligenes, Cytophaga, Erwinia, Leuconostoc, Pseudomonas, Staphylococcus,* and *Vibro.*" The reason why such organisms would not survive is not clear. It would seem that the paraffin-oil storage method is generally useful, but that it should be tested out for the particular group of stocks concerned. Little or no mention is made of the culture medium to be used, but it might have a bearing on survival. If one assumes slow growth during storage, the medium should be so chosen as not to be exhausted of nutrient and not to accumulate excessive acidic products; in general, a well-buffered medium, such as a rich protein digest with minimal sugar content, would be indicated.

Soil-Stock Method

A second, rather simple method for preservation of cultures may be considered for those organisms for which it is advantageous, i.e., dry spore stock on sterile soil. It is difficult to trace the origin of this method for stock cultures. It was reported by Bredemann[3] that passage of *Clostridium pasteurianum* on sterile soil would stimulate its nitrogen-fixing ability. Thaysen[28] applied the principle to stock cultures of the Weizmann butyl organism, but used acid-washed sand instead of soil, apparently to standardize the substrate. However, the use of neutral garden soil has become general in the handling of butyl anaerobes industrially. A small proportion of sand, such as 5 to 10%, is often added to prevent caking of the soil in the tube and a small amount of calcium carbonate, such as 1 or 2%, may be added with the culture suspension to neutralize acids. A word of caution is offered on sterilization of the soil. It cannot be done in a dry oven without charring the organic matter. Autoclaving is, therefore, utilized, but dry soil is very difficult to sterilize. It wets over the surface; air is trapped in the spaces; and then sterilization must proceed by conduction of heat through a aterial of very poor conductivity. It is not uncommon to require to 15 hours at 15-lb steam pressure to sterilize a basket packed vith tubes of soil or a large flask of soil. The sterilization can be peeded up and made much more certain by moistening (but not vater logging) the soil with a few milliliters of tap water, added fter the soil is placed in tubes. Steam from this internal water lrives out the air from the soil and materially hastens sterilization. However, it is always wise to sterilize at least 2 or 3 hours, at 15-lb steam pressure, with the tubes loosely held in the baskets and to test random tubes for sterility before a batch of soil is accepted for stock-culture use.

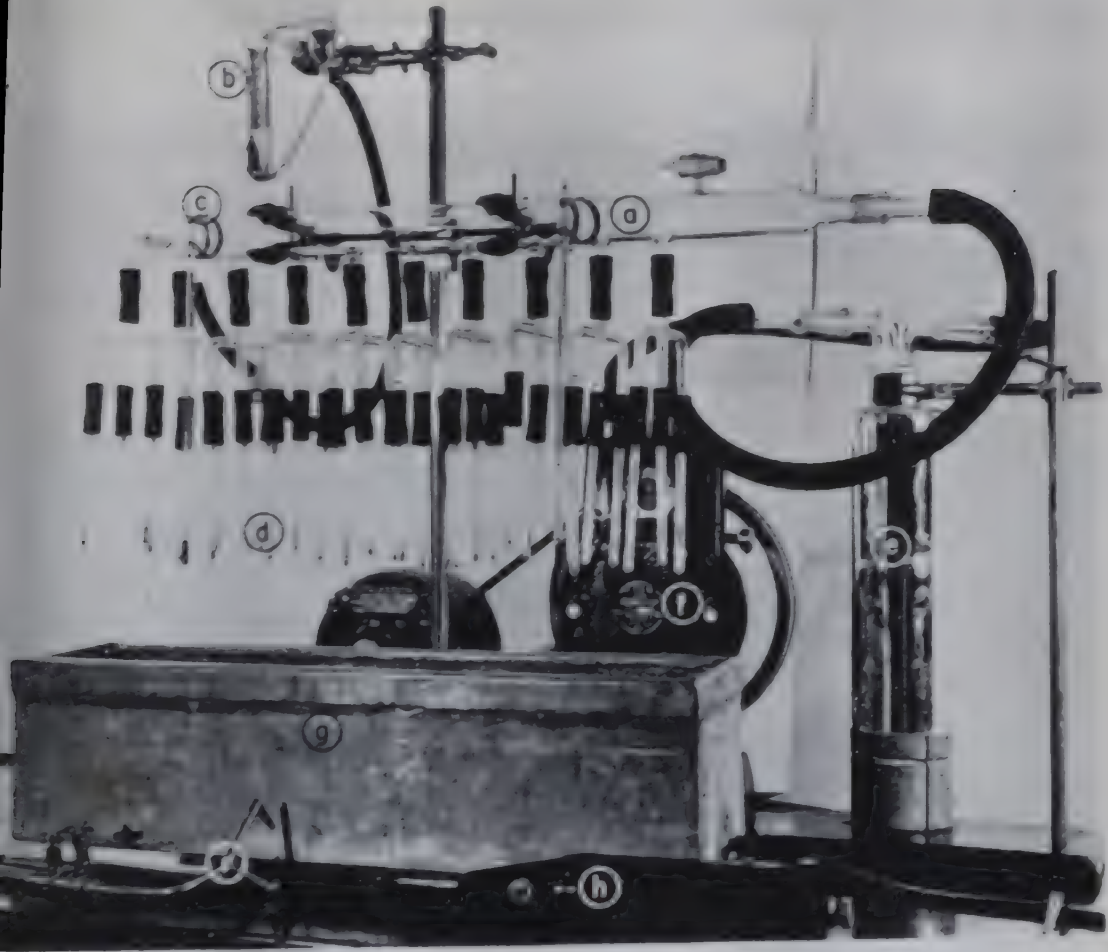
In making stocks on soil, one has to take into account whether the cultures are to grow in the soil or merely to be carried on it in dormant state. The soil for cultures of the butyl-butyric anaerobes serves only as carrier of spores. Thus a good spore stock, ripened in some suitable fermentation medium, must first be prepared. When the spore count is high, as judged by number of free spores, and when the spores are properly ripened, perhaps 2 or 3 weeks of age, the spore stock can be made. Enough volume of the spore suspension is added by pipette to saturate the soil. The soil tube is then dried spontaneously in air or in a vacuum desiccator, if convenient. Storage may be in air at room temperature for the anaerobes, even the thermophilic anaerobes (McClung[15]). In our experience, the butyl-butyric anaerobes may remain viable in soil stocks as long as 21 years; one of the cultures, *Clostridium pasteurianum*, was tested physiologically for nitrogen fixation and fermentation balance on glucose-yeast medium and was found unchanged from laboratory records on the same culture 12 years earlier.

The soil-stock method lends itself well to the preservation of mold cultures (Greene and Fred[11]) and of streptomycetes (Jones[15]). Both molds and streptomycetes grow in the soil, as can be seen from the whitish mycelium filling the pores of the soil. In the Jones' technique, moist soil is employed which would surely favor massive formation of spores in the culture. No doubt, the very high numbers of spores are a factor in the survival of such cultures in storage. Jones reported "at least 4.5 years without transfer" for *Streptomyces* species. Our own experience confirms at least that duration in species. Our own experience confirms at least that duration in observations on an extensive set of *Streptomyces* species stocks; we have also the casual note of viability of some soil *Streptomyces* species at 11 years of age. Our experience with a large mold collection has been also very good. *Aspergillus* and *Penicillium* species are viable for years; certain of the cultures of Dr. Greene, prepared in soil in 1935, were tested in 1949 and found alive. Some eight or nine other genera in the stocks are also successfully preserved, but *Rhizopus* species have occasionally been lost, so that it is our present practice to keep the *Rhizopus* species in double set, one on soil and one on malt agar, the second with yearly transfer. At each transfer of the malt cultures, we test the corresponding soil stock and, if viable, hold it longer. In case one of these *Rhizopus* cultures is needed for experiment, it is taken from the soil stock which is considered most likely not to have degenerated.

The Lyophilization Method

A third general procedure, that of lyophilization of cultures, is to be highly recommended from the data in recent literature. Lyophilization involves desiccation in vacuum from the frozen state to give a product which is readily hydrated for subculture. The process has much to recommend it in principle. Protein denaturation, including destruction of enzyme systems, is minimized by the dry state which is rapidly approached. The harmful concentration of salts and other solutes resulting from loss of water is avoided by the prefreezing, and oxygen is excluded by the high vacuum involved in the procedure. Obviously, the culture must be sealed in vials under vacuum to maintain these conditions. The very fact of sealing in glass vials is considered troublesome by some, but it need not be. It is possible to use manifolds so that a group of cultures can be made at one time and the sealing itself is simple. Subculture involves breaking the seal and thus the individual culture is destroyed at the time of subculture.

There are several modifications of the lyophile process. The apparatus may be of the Cryochem or Flosdorff-Mudd type[7,8] or that of Wickerham and Andreasen,[30] or various improvisations of the principle.[9,14] Photographs of Wickerham's apparatus are shown in Figures 34 and 35. Success appears to depend on rapid attainment of the frozen state, complete dehydration, and maintenance of vacuum or inert atmosphere[20] at all times during production and storage. Even so, the lethal effects on the culture are appreciable and studies have been made to discover the cause of death of a very large percentage of cells during or soon after



IGURE 34. *Stationary Table Lyophil Apparatus* (Courtesy—Northern Utilization Research Branch)

yophilization.[9,20,26,29] Based on the observation that such natural
olloids as milk, blood serum, and mucin have a protective effect
n dried cells, Heller[13] generalized that death of cells from the
esiccated state was greater in a menstruum containing crystalline
ompound of high solubility, which the organism could metabolize.
onversely the percentage survival was greatest in presence of
rotective colloids of low gold number and high hydrophilic prop-
rty. Peptone, normal rabbit serum, and "broth" served very well
n this capacity. Actually for stock-culture purposes, one is not
ecessarily interested in the highest percentage survival during
yophilization and subsequent storage, but it may be a factor in the
uccessful subculture from the stock at later time. There need be
nly sufficient numbers surviving *in all cultures* to assure 100%
uccess when the transfer is made. It is general practice to use the

whole dried mass as inoculum in subculture. That lyophilization does provide such a high percentage of positive subcultures is the rule. The method is recommended for bacterial cultures by Bushnell,[4] for yeasts by Wickerham and coworkers,[30,31] and for fungi by Raper and Alexander[21] and by Fennell, Raper, and Flickinger.[6] It may well be considered by any laboratory that plans a very large collection of cultures or has need of a large number of vials of any one culture for use as permanent and stable stock of its starter culture.

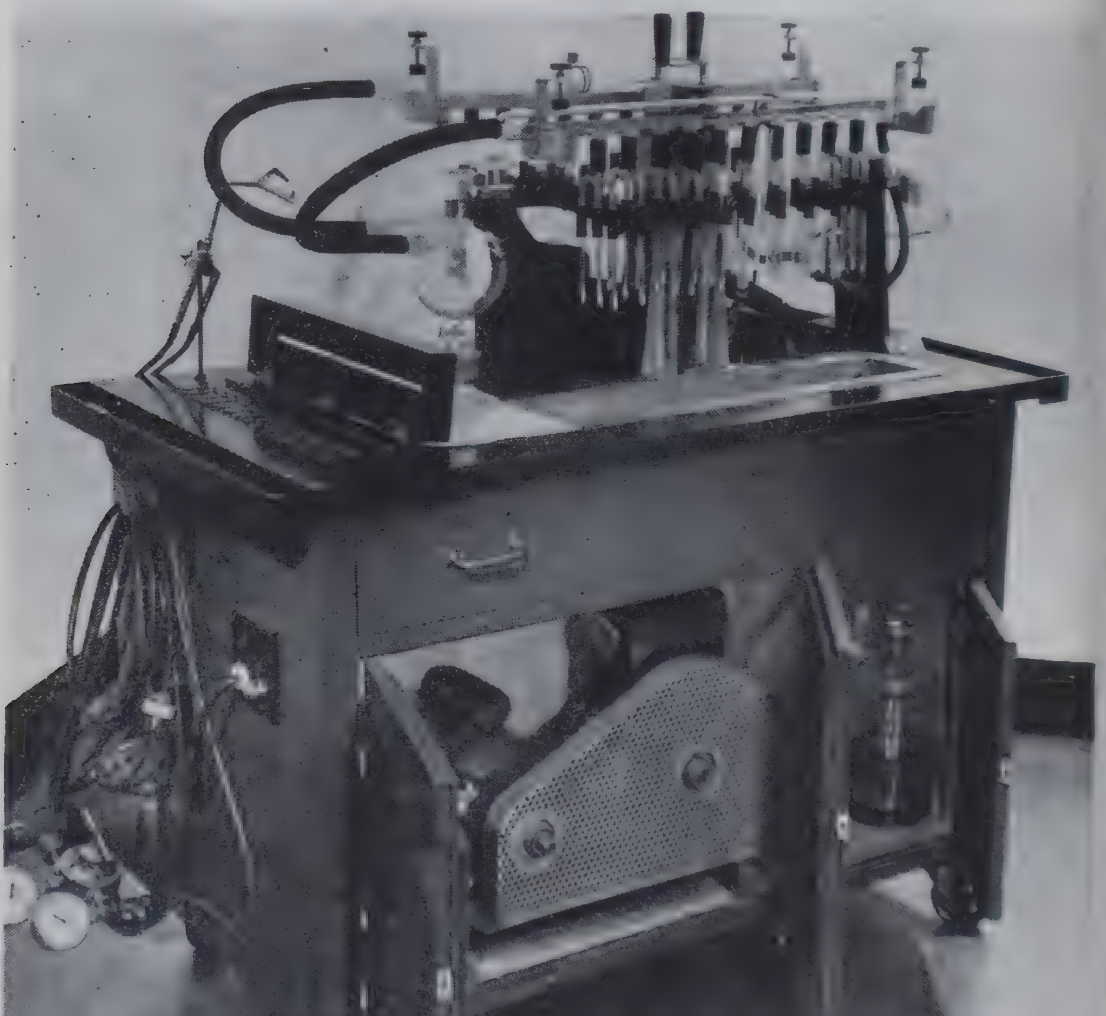


FIGURE 35. *Completely Contained Mobile Lyophil Unit* (Courtesy —Northern Utilization Research Branch)

The stability of physiological characteristics is generally considered good for lyophilized cultures,[6] but there are a few reports of change. Nymon, Gunsalus, and Gortner,[19] after trying lyophilizaon of microbiological assay strains of *Lactobacillus arabinosus* and . *casei,* reported that the first maintained its original acid-pro- ucing capacity whereas that of the second decreased somewhat, ut remained stable afterward. Stanier, Adams, and Ledingham[27] dvocated lyophilization to stabilize the highly variable *Bacillus olymyxa* in the desired phase and in high-yielding state for 2,3- utanediol fermentation. However, marked changes may follow yophilization in certain cases. Atkin, Moses, and Gray[1] found hat certain brewers' yeasts underwent changes in vitamin require- nents, suggestive of mutation or segregation of characters. It should e noted that a very large proportion (99.98%) of the cells of their cultures failed to survive lyophilization. One might speculate that the "changed" cultures might represent minor types within the parent population, which for reasons unknown proved more resistant to lyophilization. At least there is no apparent reason why lyophilization should act as a mutagenic process and this case involving yeasts is the only such report.

In conclusion, it may be said that there is available a variety of methods for maintenance of stock cultures, no one of which is recommended to the exclusion of the others. The choice may be determined by available apparatus or, if all are available, by experience with them for the cultures to be stocked.

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CHAPTER 13

CULTURAL VARIATION AND GENETICS

Carl C. Lindegren

Cultural variation in microorganisms occurs so abundantly that it is debatable if any such thing as a "pure" culture exists or can be maintained. This is not merely an academic question, but one of great importance to practical workers interested in reproducing fermentations or biological syntheses. Frequent single-cell isolation is often proposed as a means of maintaining pure cultures. Actually, this may have exactly the opposite result by selecting variants that may differ radically from the population which makes up the culture. Therefore, the maintenance of "pure" cultures will not be discussed in this chapter, but rather procedures which will maximize or minimize cultural variation.

GENERAL PRINCIPLES

Minimizing Cultural Variation

There are two empirical rules governing cultural variation of microorganisms: (1) Variation can be minimized by frequent transfer (just before full growth is attained), using large inocula on a uniform good medium (or simply on a uniform medium). (2) Variation can be maximized by infrequent transfer, using small inocula on a variety of deficient media.

There is, however, no technique for eliminating variability. It can only be minimized by providing nutrients which will insure the *continuity* of cell structure in the culture resembling as closely as possible the cells from which they were produced. *Continuity*, however, does not mean *identity*. No new cell is exactly like its progenitor and the operator can only insure continuity of the culture. The similarity of organisms of the same species which we isolate from nature is not evidence of the stability of the hereditary apparatus of the species. It is merely evidence of the response of the organism to its environment and of the selectivity of the natural environment for the specific type of organisms. When we transfer the culture to artificial environment, the inherent variability (which was concealed by the operation of natural selection under natural conditions) becomes evident.

The principal bases for the validity of these rules are:

(1) In organisms with complex life cycles (especially if two or more mating types are involved), *large inocula* are essential to avoid separating different mating types or other essential components of the life cycle.

(2) *Frequent transfers* are essential to avoid selecting specialized cells, such as spores which survive after the death of most of the vegetative population.

(3) A good medium is necessary to insure the continued growth of all the forms present in the original culture.

(4) A uniform medium is necessary to prevent selection of specialized types different from the original culture. This uniform medium need not necessarily be a *good medium* if only one specialized type of cell is to be propagated. Then a medium capable of favoring this type and unfavorable for other variants can serve to maintain the type culture. However, frequent transfers of large inocula are still required to avoid the selection of other variants.

(5) Variation either due to gene mutation or transmissible extrachromosomal change (Dauermodifikation[15]) may occur in the vegetative phase and large inocula on uniform good medium are necessary to avoid selecting and separating variants of this type from the mixed population which makes up the culture. Large inocula are also necessary to preserve the *mutational or adaptive potential* of the population. For example, Luria and Delbruck[37] showed that by inoculating about five hundred cells of colon bacillus into 0.5 ml of medium, one could obtain a crop of cells which

ontained either very few or a great many cells capable of resisting acteriophage lysis. Shapiro[41] showed, however, by calculation from hese results that an ordinary large inoculation would insure that a tatistically uniform fraction of the population would be capable f resisting bacteriophage lysis. Large inocula insure the transfer f a sufficient number of those mutants which occur with a regular requency to maintain an adequate population of the mutants and f the culture is carried for the *mutant* (rather than the cell type rom which the mutant arises), it will be present in large inocula.

Life Cycles

Understanding of cultural variation requires a knowledge of the life cycle of the organism. Life cycles are divided into two principal phases with regard to chromosome number: (1) the haplophase, with a single set of chromosomes in each nucleus and (2) the diplophase, with pairs of chromosomes present in each nucleus. Diplophase nuclei are produced by the fusion of two *haplophase* nuclei. The haplophase is produced by the "reduction" of a *diplophase* nucleus by two consecutive nuclear divisions which finally results in the production of four haplophase nuclei. In "reduced" haplophase nuclei, the gene complexes have been reorganized (1) by random assortment of the different chromosomes and (2) by crossing-over, interchange between pairs of homologous chromosomes. In Figure 36 the single lines represent the haplophase and the double lines, the diplophase. Life cycles are sometimes diagramed as circles with the implication that after a series of operations, the same kind of individual that was originally present is reconstituted. However, no two individuals are exactly alike and all biological systems are changing with time from a former condition to a future one. Stability in a biological system is based on continuity in time, but this does not imply identity of the individual organisms.

Figure 36 shows some of the possibilities when a medium is inoculated with a diploid yeast cell. The sidetrack indicates the reduction of one of the diploid cells. Each of the crossbars indicates a cell division. The continued vegetative multiplication of the original diplophase culture does not mean, however, that all of the offspring are identical since the environment is being changed constantly by the continued growth of the cells themselves. These new diploid cells may have nearly the same total *genetical potentials* as their forbears, but this potential has been reassorted and the cells have undergone differentiation (Dauermodification) by the change in the environment. That close genetical relationship does not assure identity is obvious when we consider that the highly differentiated cells in a metazoan are all descended from a single original diploid cell.

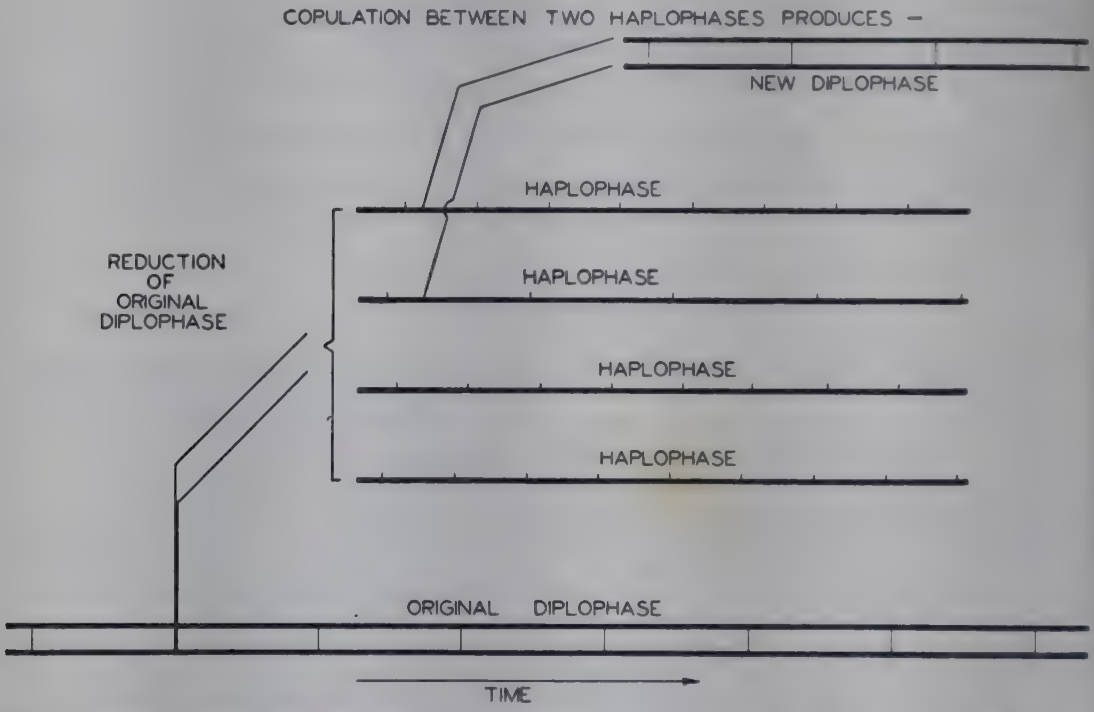

COPULATION BETWEEN TWO HAPLOPHASES PRODUCES –
NEW DIPLOPHASE
HAPLOPHASE
REDUCTION OF ORIGINAL DIPLOPHASE
HAPLOPHASE
HAPLOPHASE
HAPLOPHASE
ORIGINAL DIPLOPHASE
TIME

FIGURE 36. *Life-Cycle Diagram Illustrating the Effects of Reduction and Copulation in Increasing the Complexity of a Population*

Competition

In the culture indicated in Figure 36 the clone of differentiated diplophase cells competes with the four haplophase cultures descended from one of the diploid cells, as well as with the other diplophase and haplophase clones. Each cell has its own inherent rate of division or "biotic potential," but it is influenced by and itself affects the rates of division of all its neighbors by the effect it has on the food supply and accumulated waste products. Competition is the most potent factor controlling the characteristics of the culture. A newly constituted diplophase of high vigor may end to overgrow and crowd out its weaker relatives. However, conditions are changing so rapidly and are so nonuniform, even in a small volume, that no *one clone* can be the best adapted and thus a variety of genotypes is maintained.

Functions of the Sexual Mechanism

There are two different phases in the life cycle of a microorganism, the sexual phase and the asexual phase.[6,8,23] The survival of the sexual mechanism lies in the opportunity which it provides the organism for reassortment and recombination of hereditary characteristics. Recombinations usually result in the reconstitution of the wild type and the mutants usually fail to survive in competition since the wild type is adapted to the natural environment. Lederberg and Tatum's[17] work on *Escherichia coli* describes a mating of two mutants, each characterized by different vitamin deficiencies, which produces a small number of "prototrophs" in which the original nondeficient stock is reconstituted. Presumably, all the other possible recombinations are also made, but the prototrophs are most easily detected for they do not have any of the vitamin deficiencies and can grow on unenriched medium. In nature, the sexual mechanism often reconstitutes a *type of hybrid* better adapted to changed conditions. In wheat rust, the sexual cycle is confined to the barberry plant. Whenever plant breeders develop a new type of wheat capable of resisting the current rust, new combinations are produced on the barberry capable of attacking the resistant wheat.

The continuance of the sexual mechanism depends on the maintenance of fertility.[28] The sexual reaction is so complex that any one of an enormous number of defects can prevent its continued operation. When this occurs, the organism reproduces asexually and, therefore, loses the ability to recombine its transmissible genetical characters with those of its relatives. This does not mean that it has lost the capacity for variation, since the asexual phase is still capable of a wide spectrum of genetical change, but the latitude of the variability is now restricted and may proceed in a direction quite different from what would occur if the stabilizing effect of sexual reproduction intervened occasionally.

In the haplophase, the single set of chromosomes is present in the cells, while in the diplophase, the double number of chromosomes is present.[22,25] The diplophase is formed by the fusion of two similar or dissimilar haplophases. The function of mating type, or sex, is to insure that the two fusing haplophases will not be closely related and thus to encourage fusions between widely dissimilar haplophases. The survival value of this mechanism depends on the fact that vigor often results from matings between dissimilar individuals. When haplophases are again produced from the diplophase, the hereditary factors are reshuffled and the haplophases produced are often extremely different from the two parents which produced the original diplophase. This mechanism, involving the recombination of hereditary factors, has an enormous survival value and has been demonstrated to exist in the lowest forms of life even including bacteriophages and bacteria. The opportunity for adaptation by recombination which is available to organisms capable of recombining their hereditary factors by a sexual mechanism suggests that they would outgrow closely related individuals whose reproductive devices were limited to an asexual apparatus. The diplophase is often an inconspicuous fraction of the total life cycle and this fact has sometimes led to the conclusion that it is absent. *Neurospora*[8] and the colon bacillus[17] were studied for nearly 100 years before the discovery of their sexual stages, but in both of these organisms, the inconspicuous diplophase has been the principle factor in producing the recombinations required in their evolutionary development.

In bacteriophage, bacteria, fungi (except yeast), and some algae, the haplophase is the predominant phase of the life cycle. In higher plants and animals, the diplophase is the predominant phase of the life cycle. The evolution from predominant haplophase to predominant diplophase is the result of the requirement of organisms with complex structures for a maximum stability of the cells which act as building blocks for the complex structures. The absence of complex structures in lower organisms makes possible the extensive growth in the haplophase with the accompanying extraordinary variability. The variability may lead to sterility of a small fraction of the haplophase and prevent the sterile forms from recombining their abilities, thus draining the sterile haplophases off into a dead end, so far as the continued perpetuation of the species is concerned.

Variability in the Haplophase

The best understood type of variation which occurs in the haplophase is gene mutation which can be recognized by the regular segregation of the mutated gene at the reduction division. Such a change may be either spontaneous or induced. X-rays, ultraviolet light, and nitrogen mustards are all potent mutation-inducing agents. Gene mutations often involve decreases in the ability of the cell to synthesize specific substances, generally enzymes, or vitamins, or other essential nutrilites.[4,5] Evolution has developed an array of synthetic mechanisms in each species essential to the continued maintenance of the organism in nature. Frequent recourse to sexual reproduction restores the integrity of these synthetic mechanisms and maintains the wild-type characteristics which enable the organism to compete successfully in its natural environment. The combination of abilities which are essential to the normal wild organism are not necessarily essential, nor even desirable, in a cultivated plant or animal. A desirable cultivated plant like maize, or a domesticated animal, like the milch cow might be incapable of survival under natural conditions, because the genetical changes which have been selected operate against the organism in a state of nature. In microorganisms, the loss or imbalance of a function may also prove desirable for a cultivated organism, although deleterious to the organism in its natural habitat. For example, the normal amount of riboflavin produced by yeast may be enormously increased by selection, but this imbalance might not be helpful to the organism in its natural environment. An increase in the amount of a given substance produced by an organism need not mean a total increase in synthetic ability, but may involve a block in some synthetic process which comprises many individual steps. Whenever any single step is blocked, the precursor accumulates and the increased amount of the precursor is the result of a stoppage of its utilization rather than a fundamental increase in its production. The acquisition of a new synthetic ability by an organism may involve an extraordinarily long evolutionary development and probably is encountered seldom in the laboratory. There are many reports of mutations by which the ability to synthesize various vitamins is achieved. These may be back mutations; a relatively complete system which has been dormant may have been reactivated by some rather simple process.

Adaptation to an Undesirable Environment

Many organisms show the ability to adapt to an otherwise undesirable environment. This may result when large inocula are introduced into the unfavorable medium or may occur during continued cultivation in a medium containing small amounts of some harmful substance.[24,35] Mutation usually involves a change in a very small fraction of the population and the overgrowth of the single original mutant cell.

Adaptive Enzymes

A cell may also become adapted by the production of an adaptive enzyme.[36] Adaptation to the fermentation of sugars has been most extensively studied. The ability to adapt may be under the control of a single Mendelian gene and an entire population containing this gene may adapt *en masse* to the presence of the substrate. Other stocks apparently incapable of fermenting the substrate may achieve the ability after long exposure due to the back mutation of the recessive gene for this ability and the selection of the individual mutant in the presence of a substrate. In the second case, a *de novo* mutation has not occurred, but a latent (recessive) gene has been reactivated.

LIFE CYCLES OF SACCHAROMYCES

Saccharomyces may serve as an example of the different types of variation which one may find in all fungi and bacteria. It differs principally from other fungi in having a predominant diplophase. The cells of *Saccharomyces* exist in both haplophase and diplophase (Figure 37). There are two mating types in the haplophase and the haploid cells of different mating type copulate to produce diploid cells. Kruis and Satava,[16] in Prague, and Winge,[43] in Copenhagen, showed that the standard vegetative cells are diploid, produced by copulation of two spores or gametes derived from spores. The diploid nuclei undergo reduction at spore formation to produce four haploid ascospores. The large, ellipsoidal vegetative yeast cell is produced by the fusion of two round haploid gametes derived from ascospores. Winge established the basic facts of this life cycle by a classical series of observations on the germination of ascospores and fusion of haploid cells. Winge and Laustsen,[44,45,46] a series of notable papers, showed that colonial characteristics, rmentative ability, and cell shape are under the control of genes hich segregate at the reduction division. They hybridized yeasts placing a haploid ascospore from one strain in close proximity an ascospore of a second strain by use of the micromanipulator. hen all conditions are favorable, the two spores fuse to produce diploid hybrid cell.

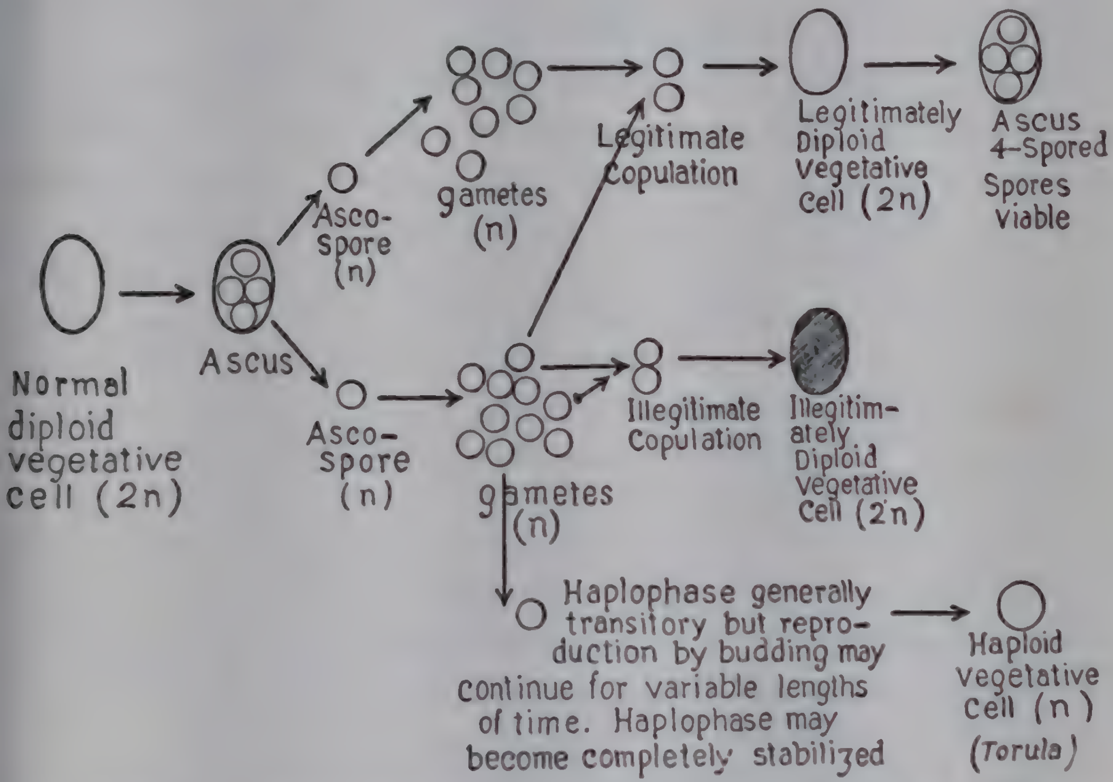

Normal
diploid
vegetative
cell (2n)
Ascus
Asco-
spore
(n)
gametes
(n)
Legitimate
Copulation
Legitimately
Diploid
Vegetative
Cell (2n)
Ascus
4-Spored
Spores
Viable
Asco-
spore
(n)
gametes
(n)
Illegitimate
Copulation
Illegitim-
ately
Diploid
Vegetative
Cell (2n)
Haplophase generally transitory but reproduction by budding may continue for variable lengths of time. Haplophase may become completely stabilized
Haploid
vegetative
cell (n)
(Torula)

FIGURE 37. *Life Cycle of* Saccharomyces (Reprinted by permission from *Ann. Rev. Microbiol.*, **1948**, 58)

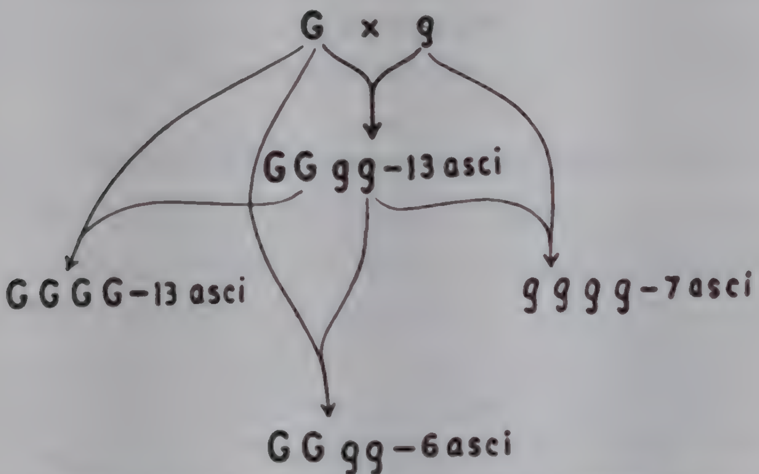
Lindegren and Lindegren[30] developed a method for hybridizing yeasts based on the fact that single ascospores of *S. cerevisiae* usually produce persistently haploid cultures. It is possible to hybridize these with other similarly derived haploid cultures by mixing the cells together in an appropriate medium. The mixtures result in copulation if each culture is paired with a complementary type. One parent culture can be mated to a large number of other clones. The parents can be classified for biochemical and other characteristics prior to the matings and the haploid progeny can be classified subsequently. Lindegren and Lindegren[30] have developed stocks heterozygous for genes controlling the fermentation of galactose, sucrose, maltose, melibiose, and raffinose. They have also developed stocks differing in their abilities to synthesize the various vitamins, amino acids, and nucleic acid components. These cultures are all interfertile and over two thousand hybrid asci have been analyzed. Several cases of linkage have been discovered and maps have been constructed.

Lindegren and Lindegren[34] discovered that haplophase cultures of *S. cerevisiae* fall into two mating types which they have designated *a* and ∝. Each haploid culture is made up of potential gametes which can be mated with other cells of appropriate genetic composition from another culture. As the pure haplophase cultures age, illegitimate copulations occur in some cultures between two cells of the same reaction in the same culture. Diploid cells produced by illegitimate copulations are generally distinguished by diminished ability to produce four-spored asci containing viable spores. Legitimate matings between *a* and ∝ haplophase cultures derived from a variety of strains of *S. cerevisiae* usually produce diploid cells which sporulate to form four-spored asci containing viable spores. The procedure is as follows: 1 ml of broth is placed in a test tube. The broth is first inoculated with a large loop of cells from an agar slant of the tester culture and shortly after with a large loop from the haploid culture to be tested. Copulations sometimes appear within 6 hours. The tube is kept overnight in an incubator at 19°C, and inspected under the microscope for the presence or absence of copulations.

Some haplophase mutants obtained by continued selection involving numerous platings and transfers over a prolonged period (usually more than 1 year) were found to be incapable of copulation. Prolonged competition and selection probably result in loss of fertility because the genes which insure fertility do not have a high survival value in competition with other rapidly growing mutants.

Mendelian Inheritance

Figure 38 summarizes data on the inheritance of a Mendelian gene controlling the adaptive fermentation of galactose. No exceptions to standard Mendelian inheritance were found. Thirteen asci were analyzed from a heterozygous hybrid made by mating a galatose fermenter (*G*) by a nonfermenter (*g*); two spores in each of these asci carried the dominant gene controlling fermentation of lactose and two carried the recessive allele. A backcross of fer- enter to the fermenter parent produced thirteen asci; all four ores in each of these asci carried the fermenting gene. A back- ross of the nonfermenter to the nonfermenting parent produced even asci, each of which contained four nonfermenting spores. A eterozygous zygote was produced by backcrossing a nonfermenter the fermenting parent; six asci were analyzed and each contained wo fermenting and two nonfermenting spores. This analysis shows uite convincingly that in some pedigrees the genes controlling ermentation of galactose may behave in a regular Mendelian nanner.



IGURE 38. *Mendelian Inheritance of Galactose Fermentation in* Saccharomyces (Reprinted by permission from *Ann. Rev. Microbiol.,* **1948**, 60)

Non-Mendelian Inheritance

In contrast to the regular Mendelian behavior of genes con- rolling galactose fermentation described before, irregular segre- ations have also been discovered in yeast.[26,33] Some instances of rregular segregations comprise *bona fide* examples of non-Men- lelian inheritance due to gene conversion. On this view, a chromo- ome may carry a synthesizing mechanism which can be transferred rom an active to an inactive chromosome or can be lost from an ctive chromosome. Some irregular segregations are due to changes n ploidy. Heterozygous hybrids (*G/g*) have produced ascospores which are diploid and heterozygous for the character in question. Outcrossing the diploid ascospores has produced triploid and tetra- loid zygotes.

Yeast cultures produce some variants which are not gene mutations, since their characteristics cannot be transmitted through the sexual cycle. We have thus confirmed Jollos'[15] concept of the Dauermodifikation. The variants derived from vegetative cultures are stable when propagated vegetatively but when the variant is mated with a normal culture only normal offspring are produced. The variant strains[40] are usually characterized by smaller colony size, loss of red pigment (if the parent culture was red), absence of certain cytochrome oxidase enzymes, or inability to utilize alcohol. These defects may all be the result of the same basic extra chromosomal deficiency which can be restored to normal by the outcross. This study has suggested that a considerable degree of the variation encountered in microorganisms is not due to gene mutation but is the result of a defect in some structure other than the chromosome.

Haplophase Variability versus Diplophase Stability

Nearly all haplophase yeast cultures, when plated on agar produce numerous relatively stable colonial "mutants," while the corresponding diplophase cultures only rarely produce colonial variants. Hybrids are produced by mixing the cells of two apparently unstable haplophase cultures together in a small amount of broth. After copulations occur, diploid cells are produced which subsequently sporulate. Genetical analyses of the hybrid are made by dissecting four-spored asci at random after sporulation has occurred. One of the puzzling things about this process was the fact that in spite of the considerable potential of the haplophase cultures for variation, analyses of the offspring from matings usually yielded surprisingly regular results. This was supposed at first to be the result of statistical sampling; in the enormous population of cells, the chances that a given zygote had been derived from the two preponderant genotypes is presumably very great. However, the fact that Dauermodifikationen occur so frequently suggests that many of the variants which appear on the plates cannot be transmitted through the zygote. The reliability of the method of mass mating is greater than the variability of the haplophase would lead one to believe and numerous trials have confirmed this conclusion.

Segregation

Segregation of genes occurs when the chromosomes of the diploid cells are segregated at the reduction division just prior to

orulation. The haplophase originates by the reduction of the
plophase at spore formation and the segregation of a heterozygote
oduces segregants of different genotypes. Each of the four spores
rmed in a single ascus is usually genetically different. The
ploid segregants are usually rough colonied. The segregant
ltures also vary in fermentative ability, in ability to synthesize
nino acids, vitamins, and nucleic acid components, in color, and
the size and shape of the haploid cells. The type of cell aggrega-
on is also characteristically different. Haplophase clones generally
end to produce aggregated or agglutinated cells much more fre-
uently than diploid clones.

Haploid yeast cells are much smaller and more variable than
iploid cells, varying more than diploid cells both from culture to
ulture and within a single culture. These differences are also re-
ected in the colonies. Haplophase yeasts are nearly always inferior
synthetic ability when compared quantitatively, or qualitatively,
the diploid parent from which they originated; many of them
ave lost certain specific characteristics.

Variations in the haplophase enormously increase the number
f colonial forms, but the original segregant can generally be dis-
inguished from the secondary variants when the culture is plated.
t first, the variants are usually slow growing and produce small,
ound colonies, but on transfer, they become adapted and stabilized
nd their specific colonial character becomes apparent, distinguish-
ng them from the original segregant. Whether there is a process
nvolving several genetical steps has not been determined. Varia-
ions occurring in the haplophase can be selected and propagated.
n spite of the wide variety of types that occur, the existing genotype
f any cell limits its potentialities and the range of its possible varia-
ions. This fact has been especially brought out in experiments
imed at adapting haplophase by selection. *S. cerevisiae* is unable
ferment melibiose and prolonged exposure of haplophase cul-
ures of *S. cerevisiae* to melibiose failed to produce any mutants
apable of fermenting this sugar. A haplophase variant of *S.*
erevisiae incapable of fermenting galactose could not be induced
hrough a 4-month period to produce mutants capable of ferment-
ng galactose, although this strain produced an abundance of
olonial variants during the same period. Although "losses" occur
asily, "gains," as in the case of specific fermentative or synthetic
bilities, apparently do not occur at all under certain conditions.

Recombination

Segregation and mutation produce a great variety of haploid gametes. Copulations between these gametes selected at random produce new recombinations which may differ in a number of ways from the diploid from which they originated. Genetical variations in yeasts are, therefore, produced by (1) segregation, (2) vegetative variation (gene mutation and Dauermodifikation), and (3) recombination.

LIFE CYCLE OF NEUROSPORA

Neurospora crassa may be studied as a type specimen for the higher ascomycetes since its life cycle is so well known. Shear and Dodge[42] named the genus *Neurospora,* which contains both eight- and four-spored species. Dodge[8] discovered that the eight-spored species were heterothallic, while the four-spored species produced homothallic spores, due to the inclusion of one nucleus of each mating type in the spore. The life cycle shown in Figure 39 is practically the same as that of *Penicillium, Aspergillus,* and similar forms.

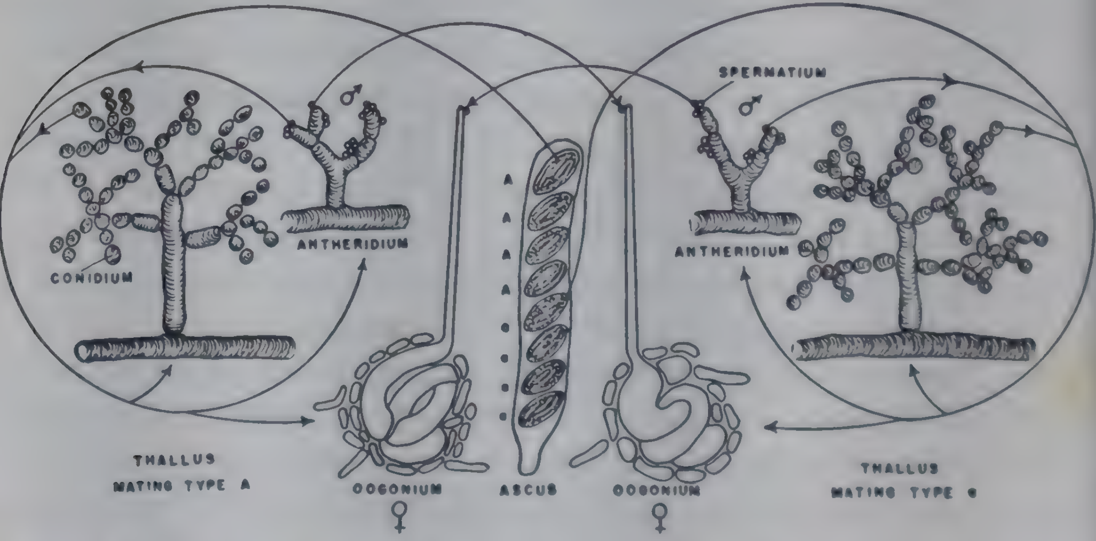


FIGURE 39. *Life Cycle of* Neurospora crassa (Reprinted by permission from *Ann. Rev. Microbiol.,* **1948**, 64)

The perithecium of *Neurospora crassa* produces a large number of asci, each containing eight spores. The spores fall into two categories with regard to mating type. Each spore gives rise to a thallus which produces a mycelium, on which male and female sex organs, as well as asexual conidia, are developed. The female sex gan is called a bulbil and contains an oogonium and trichogyne. he male sex organ produces sperm called spermatia. The spermatia from an *A* type thallus are incapable of fertilizing the ogonium developed on the thallus of the same type, but will crossrtilize an oogonium of a complementary mating type. The supernposition of sex organs and mating type shows that mating-type enes are self-sterility alleles and bear no relation to either sex rgans or the evolution of sex.

Single ascospore cultures[29] are characterized either by morphoogical or biochemical properties and hybrids are made either by ransferring spermatia from a tube of one mating type to a tube of he complementary mating type or by inoculating a single tube with onidia from each culture and allowing them to grow together.

COENOCYTIC FUNGI

Although the hyphal threads of the *Ascomycetes* are divided nto chambers of cross walls, these septations are practically always erforated and function to keep the tubes from collapsing rather han to separate the organism into cells. The genetic mixture of aploid nuclei suspended in the common cytoplasm passes freely hrough the septations as the cytoplasm streams along the hyphal hreads. If one of these haploid nuclei mutates, the mutant may ontinue to increase by division and become distributed throughut the plant by cytoplasmic streaming. The genetical heterogeneity f the nuclei prevents such an organism from producing structures f great complexity; in fact, in these organisms, genetical complexity s maintained at the expense of the ability to produce complex tructures. In some species, there are elaborate adaptations which ssure that the sexual spores (which contain only a single type of ucleus in most species) will contain two genetically different kinds f nuclei.

Hyphal Fusion

Hyphal fusion or anastomosis is a device which functions to reserve and increase the genetical complexity of the fungi. When wo hyphal tips come into contact, their contiguous walls dissolve nd their contents intermingle. When a hyphal wall becomes old nd thick, it is incapable of fusion. These fusions have no sexual ignificance and are not followed by karyogamy. They occur beween mycelia of the same mating type as frequently as between mycelia of opposite mating types. They sometimes occur between genetically different hyphae and may even occur between hyphae of different species or genera.

Heterokaryosis

The facility of nucleo-cytoplasmic interchange produces a mixture of genetically different haploid nuclei. This condition is called heterokaryosis and the heterokaryotic thallus is the biological equivalent of the diplophase in other forms. When masses of spores are transferred, the young germ tubes fuse with one another as soon as they emerge from the spores and when the spores are genetically different, the new mycelium contains a mixture of different kinds of haploid nuclei. An interesting result of this capacity to produce nuclear mixtures is the fact that lethal genes in haploid nuclei can be carried in culture because the normal nuclei in the cytoplasm provide a sufficient excess of the substances necessary for subsistence. The fact that the "weaker" pseudo-parasitic or even lethal mutant nuclei can be supported by the more vigorous normal or wild-type nuclei means that practically all new mutations are preserved and live in the cytoplasm elaborated by the more vigorous "normal" hosts. Since a gene is only "poor" or "good" in certain combinations, the low vigor of a genotype does not mean that the gene itself is not valuable for the species. The new genotype multiplying passively in the host cytoplasm recombines with other mutants in the sexual cycle. If a genotype of exceptional vigor or specific adaptability is eventually produced, it may attain supremacy in the heterokaryon and finally produce sectors of new growth. Mutations in diploid cellular plants rarely attain supremacy in the plant in which they occur. Mutations which occur in somatic cells, even if they are dominant, are walled off from the rest of the plant and fail to affect its character unless they occur early in a bud (and cause a bud sport). An advantageous mutation in a fungus thallus, however, because of the coenocytic character of the plant, has a good chance of dominating the thallus, although it can rarely become completely separated under natural conditions from the mixture of nuclei from which it arose.

Some of the diverse nuclei found in fungus thalli are capable of performing specific functions. For example, in *Neurospora*, forms found in nature may be heterokaryotic for normal conidial and nonconidial mutant nuclei. These nonconidial mutants are in-apable of producing the asexual spores (conidia) by which the ungus is disseminated vegetatively. Since they do not expend nergy in the formation of asexual spores, they grow more rapidly han the normal conidial forms. The heterokaryon has the rapid rowth character of the nonconidial mutant and the conidium-orming capacity of the conidial form and is, therefore, more vigorous than either pure form. It is conceivable that mixtures of nuclei re important in other ways not so easy to demonstrate. A heterokaryon carrying mutants capable of elaborating different kinds and amounts of enzymes would probably have a higher survival value than a more restricted and less flexible homokaryotic form.

Organisms, such as fungi and bacteria, in which single thalli or colonies contain mixtures of genetically differentiated nuclei, can possess only a minium of differentiated structures and in place of structures capable of performing a variety of functions, genetically different nuclei may be specifically adapted for the performance of different functions. In higher plants, which are genetically uniform and protected against variation by diploidy, different functions are performed by structures differentiated morphologically from the other cellular tissue of the organism. Fungi, such as the *Hymenomycetes* (mushrooms and toadstools) which produce relatively complex structures, are not coenocytes, but are built of cells each of which contains a stable dikaryon which is duplicated by synchronous "conjugate division" of each of the nuclear mates. In mushrooms, each cell contains two nuclei and this pairing insures stability against the possibility that variation might produce a new phenotype and distort the structure. Stability is, therefore, assured by dikaryons in *Hymenomycetes* just as it is assured by diploidy in the cells of higher plants. However, a *Hymenomycete* dikaryon is an extremely flexible structure and one of the members of a dikaryon is often replaced by an "invading" nucleus which is genetically different. Smut mycelia capable of invading host tissue and causing disease are dikaryotic. Monokaryotic strains of smuts are usually incapable of parasitism, while the dikaryotic bisexual mycelium easily gains entrance to the host tissue.

Asexual Cycle

Asexual spores (conidia) of *Neurospora* are usually multinucleate, thus insuring the vegetative propagation and dissemination of the heterokaryon existing in the thallus. When a conidium is finally separated from the conidiophore by a solid wall, it may contain a few to a dozen or more haploid nuclei. In nature, the variety of mutant nuclei present in a single conidium usually contains representatives of each of the two mating types. A conidium planted on agar produces a germ tube which branches rapidly and grows radially from the point of inoculation, with simultaneous multiplication of the various nuclei.

The mycelium is made up of branching tubes divided into cylindrical compartments by septa (cross walls), but these cross walls do not separate the threads into cells in the regular sense. Two partitions may enclose a mass of cytoplasm containing any number of nuclei, from a few dozen to many hundreds, each of which is haploid. Furthermore, each septum has a large perforation at the center and nuclei migrate freely through the perforations. When the hyphae have attained nearly full growth, an abundant deposit of nuclei and cytoplasm has accumulated. During the early stages of growth, only a few conidia are produced, but finally, when the growth of the hyphal mat nears its limit, conidia are formed in abundance.

The large hyphal tubes in the center of the mat are completely emptied of their nucleo-cytoplasmic mixture and, as the emptying progresses, a plug is formed at each septal perforation. (Sometimes a thin hyphal thread will grow back into the large, emptied hypha through the perforation.) The abundance of already elaborated cytoplasm and nuclei is stuffed like the filling of a sausage into the terminal hyphae, which now take on a predominantly aerial growth, probably stimulated by the staling of the substrate. The aerial hyphae branch dichotomously and the conidia are cut off by walls as the streaming into the tips continues.

Sexual Cycle

The sexual spores of *N. crassa* are black, lemon-shaped ascospores with longitudinal markings.[22] After a heat treatment at 60°C for an hour or at 90°C for a shorter time, the ascospores put out germ tubes from pores at each end of the spore. In nature, the spores lying dormant in the ground usually germinate after a fire. Before any green vegetation gets started, the aerial hyphae covered with salmon-pink conidia (asexual spores) cover the burnt-over region. The mold sometimes completely obscures the object on which it is growing. The growth is so thick that conidia can be picked up by handfuls from the surface of bread or sugar-cane bagasse. Slight disturbances of the air release clouds of conidia to spread the infection. Finally ascospores are produced as a result of the sexual cycle and the ascospores may lie dormant in the soil for several years until the next fire occurs. The asexual spores usually die in a few months.

In the laboratory, it is possible to obtain pure cultures from the ascospores of *N. crassa* by planting them on agar and subjecting them to a heat treatment. Heat treatment kills the vegetative mycelia and asexual spores and stimulates the ascospores to germinate. After the ascospores germinate, a small block of agar containing a single spore is cut out and transferred to a culture tube. Such cultures contain an abundance of vegetative mycelia and asexual spores, but no ascospores appear in a culture grown from a single ascospore.

The fungus is heterothallic, producing **A** and **a** ascospores. Cultures from either **A** or **a** spores look exactly alike to the naked eye and under the microscope. When **A** and **a** mycelia are planted together in a tube, perithecia filled with ascospores are produced.

The male gametes are called spermatia. They are produced on both **A** and **a** mycelia. The spermatia are small (3 to 4 μ), thin-walled, uninucleate cells which act as sperm. They are extruded from a pore at the tip of a bell-shaped hypha or from pores in the sides of short, stubby hyphae (spermatiophores). **A** spermatia only fertilize **a** cultures, and **a** spermatia can fertilize only **A** cultures.

The bubils are the female sex organs. Each contains an oogonium tightly wrapped in a dense ball of thick-walled hyphae. From the oogonium, a branched trichogyne extends sometimes for a long distance. **A** bubils are transformed into perithecia containing asci full of ascospores if they are spermatized by spermatia from an **a** culture. Bubils which are indistinguishable from each other are found in both **A** and **a** cultures.

Segregation in the Ascus

The ascospores are contained in the slender tubular asci inside the perithecium. From fifty to one hundred are normally found in each perithecium and each ascus contains eight spores.[18,21]

Each ascospore is binucleate, but the ascospore at its origin contains only a single nucleus; the paired nuclei are genetically identical and the culture produced by growing a single spore is homokaryotic. Four of the spores from each ascus are **A** and four are **a**. The zygote is produced by a nuclear fusion occurring in the young ascus. One haploid **A** gametic nucleus and one haploid **a** gametic nucleus always participate in the fusion. The zygote immediately undergoes reduction and the distribution of the two types of spores in the ascus is due to the reduction of an **A**/**a** gene pair in the zygote. The fact that (1) the zygote is always heterozygous for the **A**/**a** gene pair, (2) that no zygote can be produced unless it is heterozygous for these genes, and (3) that every member of the species (and probably the genus) falls into either the **A** or **a** category with no alternative class, differentiates these mating type alleles from other sterility factors. Sterility factors are well known in *Neurospora* and have been extensively studied, but cannot possibly be confused with the sex factors. We have, therefore, two mechanisms involved in producing the zygote: (1) the sex organs, spermatia (male) and bulbils (female), and (2) the mating type factors, **A** and **a**, for which the zygote is always heterozygous. The two kinds of spores are arranged in the ascus in six ways:

Arrangement	1	2	3	4	5	6	7	8
(1)	**A**	**A**	**A**	**A**	**a**	**a**	**a**	**a**
(2)	**a**	**a**	**a**	**a**	**A**	**A**	**A**	**A**
(3)	**A**	**A**	**a**	**a**	**A**	**A**	**a**	**a**
(4)	**a**	**a**	**A**	**A**	**a**	**a**	**A**	**A**
(5)	**A**	**A**	**a**	**a**	**a**	**a**	**A**	**A**
(6)	**a**	**a**	**A**	**A**	**A**	**A**	**a**	**a**

The first two arrangements (four and four) are the result of Mendelian segregation of the mating type factors at meiosis I. The last four arrangements (two and two) are the result of Mendelian segregation of the factors at meiosis II.

The third division is equational, so that each spore pair (1 and 2, 3 and 4, 5 and 6, 7 and 8) is genetically identical. A fourth division occurs in each spore which is also equational and results in each spore containing a pair of genetically identical nuclei.

PENICILLIUM

The discovery of penicillin has resulted in considerable interest in variations among species of *Penicillium* in penicillin production.

Derx[7] proved by mating single ascospore cultures isolated from *Penicillium luteum* that this species is heterothallic. *P. notatum* (and *P. chrysogenum*), from which penicillin is currently produced, is a form species rather than a true species. It probably originated from *Penicillium* ascospores and is perpetuated in nature asexually. The existence of heterothallic species in *Penicillium* suggests that the sexual mechanism may be exploited to obtain penicillin-producing strains. It may be significant that Derx mated the cultures which he obtained from single ascospores very shortly after having isolated them. If the same cultures had been tested a few months later, they might have been incapable of producing perithecia. A parallel situation is found in yeasts. Lindegren and Lindegren[31] have shown that haploid yeast cultures capable of copulating vigorously may lose their mating-type specificity and become completely sterile when carried in culture. Lindegren, Beanfield, and Barber[28] described variations in the fertility of *Neurospora* cultures.

Heterokaryons in Penicillium

Foster, Woodruff, and McDaniel[12] have shown that *P. notatum* becomes heterokaryotic by mutation, with a rapid loss of penicillin-producing ability. They found that the new mutants which occurred in the mat seemed to decrease its capacity to produce penicillin.

Baker[2] showed that the septations in *P. notatum* are perforated and that fusions between germinating spores and between different hyphae occur frequently. Her figures suggest that conditions in *Penicillium* may approximate those in *Neurospora*, in which heterokaryosis is extremely important, especially for survival in nature.

Hansen[13] has described the "dual phenomenon" in a wide variety of fungi and Hansen and Snyder[14] have made observations on *P. notatum*, which, they conclude, also exhibits the "dual phenomenon." What Hansen referred to is obviously simple heterokaryosis. Previously to Hansen's work, both Dodge[8] and Lindegren[18] had shown that heterokaryosis is the rule in *Neurospora* and also that the condition is not "dual" but multiple. The commonest type of mutation involves loss of the ability to produce conidia. Lindegren found at least ten different nonconidial mutants in *Neurospora*, all of which were nonallelic. To speak of a heterokaryon of conidial and nonconidial forms as a "dual phenomenon" is to oversimplify the situation. The nonconidial mutants are easy to detect by observation. This is especially true in *P. notatum* and may be due to the fact, recently discovered by Pontecorvo and Gemmel,[39] that the nonconidial form may be dominant in the heterokaryon. However, in addition to nonconidial forms, natural heterokaryons also contain a number of other types of mutants.

Lindegren and Andrews[27] made heterokaryons between different strains of penicillin-producing *Penicillia*. The first step in the formation of heterokaryons is hyphal fusion and our first experiments were designed to test the readiness with which fusions occurred between different cultures of *P. notatum* and to select strains which were able to anastomose readily. The fact that hyphae fuse has no significance from the standpoint of mating type or sexual difference. It is characteristic that some cultures are able to fuse more readily than others. In one mycelium, fusions may occur readily between adjacent hyphae, while in another strain of the same species, practically no fusions will occur.

The experimental procedure was as follows: Sterile slides were coated with a thin layer of nutrient agar and parallel streaks of conidia from a single *P. notatum* culture were made on each slide with the inoculating needles adjusted so that the points were approximately 2 mm apart. These slide cultures were allowed to incubate at room temperature (about 80°F). Conditions were optimum for observation at 48 hours. Fusions between the hyphae could be observed in the region between the parallel streaks at 48 hours, but after the second day, overgrowth generally obscured precise observations.

Three of twenty-five NRRL strains of *P. notatum* showed a marked tendency to fuse, seven others did so with somewhat less vigor, and the remainder either demonstrated a pronounced reluctance to fuse or the mycelium failed to grow across the intervening space. In very few cases did fusions occur with any of the strains where the parallel streaks were more than 3 mm apart. When the streaks were 3 to 5 mm apart, the mycelia would advance to within about 1 mm of each other and growth on the inner sides would cease, although it continued on the outside. The three NRRL strains, No. 49, No. 72, and No. 75, each of which had demonstrated a strong tendency to produce hyphal fusions with themselves, were matched in pairs on agar slides. The three possible pairings all exhibited abundant fusions after 2 days. This is exceptional, for in most other instances where the parallel streaks originated from two different strains, a pronounced barrage occurred.

When fusions occurred between two different strains, a transfer was made to an agar slant and the resulting culture was studied carefully for uniformity. In no case were sectors observed and the mycelium was always intermediate in roughness and other observable characters. Since the previous tests had shown that the mixed mycelia were compatible, we concluded that the combinations produced heterokaryons rather than fungal mixtures.

Hyphal fusion is not a mating-type or a sexual phenomenon and there is no apparent limit to the number of genotypes which can be built into one heterokaryon. A triple heterokaryon was made by mixing No. 72, 75, and 49.

Culture No. 72 (which was most compatible from the standpoint of penicillin production) was paired with two cultures which we had been using in the laboratory for penicillin production. Mat cultures of all the original strains and the heterokaryons were grown in penicillin medium in 2-qt milk bottles. All tests were run in triplicate and many in quadruplicate. The results were averaged and the agreement in all cases was rather close. Before seeding the heterokaryons, the mixed cultures were grown on agar slants. The bottles were inoculated and kept 1 day at room temperature and 7 days at 23°C. The filtrate from the bottles was assayed by the Oxford method, using glass cylinders on an agar plate sown with staphylococci. The concentrations of penicillin in Oxford units per ml are shown in Table 44.

TABLE 44. PENICILLIN PRODUCTION BY DIFFERENT STRAINS OF *PENICILLUM NOTATUM*

Strain	Penicillin units/ml	Strain	Penicillin units/ml	Strain	Penicillin units/ml
No. 49	20	No. 49	18	No. 20.4	50
72	47	72	90	40.1	5
75	25	75	22	72	33
49 & 72	12	49 & 72 & 75	2	20.4 & 72	35
49 & 75	7			20.4 & 40.1	5
72 & 75	47			40.1 & 72	27

Culture No. 49 has a definitely destructive effect on the penicillin production of cultures with which it is associated. No. 75 and

No. 72 seem to be compatible, if not complementary. No. 72 and No. 20.4 also seem compatible.

Heterokaryons in Nature and in the Laboratory

Under natural conditions, many conidia usually germinate together and if the strains are compatible, anastomoses immediately produce heterokaryons. Therefore, in nature, heterokaryosis is an especially favored condition and opportunity is afforded for continued increase in complexity of the thallus. However, laboratory manipulation of fungal cultures leads to a different end. On agar plates, single conidia germinate separately and cultures are selected which are of monoconidial origin. Many of these cultures are homokaryotic, and this is especially true of fungi, such as *Penicillium* which has uninuclear conidia. The high lability of penicillin production, as indicated by our experiments on heterokaryons, suggests that under natural conditions, where mixtures are the rule, little or no penicillin is produced. Only in the laboratory where homokaryons are handled, or when a single conidium falls in a plate (as in Fleming's original observation), can appreciable amounts of penicillin be expected. The possibility still exists, however, that the yield of penicillin can be increased by making mixtures. Dodge[11] and Beadle and Coonradt[3] showed that mutants deficient for certain physiological characteristics may mutually supply each other's deficiencies in a heterokaryon.

Because of our limited knowledge of sexuality in the *Penicillia* and *Aspergilli,* and the general conviction that with few exceptions these species are imperfect, attempts to improve penicillin production have been confined principally to the selection of supposedly imperfect cultures isolated from nature. But this program is very inflexible, for even if the isolation of a large number of different forms of *P. notatum* should yield exceptionally effective penicillin producers, improvement of the culture is limited by the potentialities of the original isolate because this so-called species apparently is incapable of mating and our own attempts at improvement by cytoplasmic mixtures do not show too much promise.

The following procedure might yield improved forms: It should be possible to find an ascogenous heterothallic *Penicillium* or *Aspergillus* with the ability to produce penicillin. Once this organism has been discovered, its sexual mechanism can be exploited to produce different segregants and those which are best from the point of view of penicillin production can be mated with each other, as well as with widely different forms. This method has been used successfully with yeasts.[32]

In *Neurospora* many, and probably most, of the isolates from nature are heterokaryotic for both the **A**/**a** alleles,[19] but sterility factors prevent copulation, so that the mycelium appears to be of one mating type. The most experienced observers may fail to detect such self-sterile heterokaryons. Lindegren[21] has reported obtaining five cultures of *Neurospora* from Dr. B. O. Dodge, which were labelled Mexico A, Cuba A, Japan A, Japan B (= a), Panama B (= a). An elaborate series of experiments was required to show that all three of the **A** strains carried two kinds of nuclei (**A** and **a**), but sterility factors prevented the production of perithecia.

Moreau and Moruzi[38] performed experiments in which a culture of *Neurospora,* freshly isolated from nature, was planted in one side of a U-tube filled with agar and a laboratory culture isolated from a single *Neurospora* ascospore (and, therefore, homokaryotic as far as mating type is concerned) was planted in the other arm of the U-tube. Under such conditions, the natural culture produced perithecia and ascospores. They proved conclusively that mycelium did not grow through the U-tube and thus only the diffusion of some stimulating substance through the agar could account for the appearance of the perithecia. The culture which had been isolated from nature was incapable of producing perithecia except under these conditions. They concluded that this experiment proved that A/a genes did not control the production of perithecia in *Neurospora.* Dodge[10] and Aronescu[1] criticized these experiments adversely, but Lindegren[20] interpreted them as indicating that the culture which Moreau and Moruzi had isolated from nature was a self-sterile heterokaryon which was made fertile by the diffusion of a substance from the homokaryotic culture. Lindegren[19] suggested that self-sterile heterokaryons occur frequently in nature among the filamentous ascomycetes because of their obvious high survival value. They enable the thallus to preempt a large area due to its rapid vegetative growth, but when vegetative growth has ceased, the conidia can fertilize other thalli with which they come in contact. The practice of plating out *Penicillia* with their uninuclear conidia breaks up any heterokaryons which may be collected. In *Neurospora* the multinucleate conidia facilitate the maintenance of the heterokaryon.

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CHAPTER 14

FERMENTATIONS IN WASTE TREATMENTS

Arthur M. Buswell

Efficient methods for disposal of wastes constitute a problem which faces almost every municipality and many industries. Since such wastes represent large volumes of liquids, they usually find their way into nearby streams or other bodies of water. However, it is generally not permissible to discharge raw wastes into natural waters, since these wastes contain organic matter which causes depletion of the oxygen below the requirements of normal fauna and flora and thus constitutes a nuisance.

Various methods have been devised by different industrial concerns and municipalities for the disposal of wastes. Waste treatment may be practiced for the recovery of valuable products and frequently, the monetary savings thus effected more than pay for the cost of treatment. In other cases, waste treatment is mandatory to diminish stream pollution. The most important processes for waste treatment involve the microbiological destruction of organic matter. Both aerobic and anaerobic processes are extensively employed and are important, but perhaps the greatest interest attaches to the anaerobic degradation of organic matter. Many of these degradation reactions, occurring under anaerobic conditions, result in the formation of methane accompanied by carbon dioxide and, in some cases, hydrogen. In this manner, nature has contrived to stabilize the carbon, hydrogen, and oxygen of which organic matter is primarily composed by the formation of these three stable products.

METHANE FERMENTATION

The formation of methane in nature has interested bacteriologists and chemists for more than 60 years. The studies in this field, commencing with a search for an explanation of the "will-o'-the-wisp," have progressed to a point where methane, enough for fuel for power plants of several thousand horsepower, is produced by anaerobic fermentation.

The literature in this field was summarized some years ago by McBeth and Scales[50] and by Stephenson.[78] Briefly, it has been known that moist organic matter, when allowed to decompose under restricted oxygen conditions, yields hydrogen, carbon dioxide, methane, and a variety of organic acids in greater or lesser amounts. Little or no quantitative data on the yield of the various products were available and little had been done with pure compounds, although Söhngen[73] had shown that lower fatty acids with an even number of carbon atoms could be decomposed by mixed cultures giving methane and carbon dioxide. Whether or not hydrogen and fatty acids were necessary intermediates in the process of methane formation was not known. The information on the susceptibility of various natural products to methane fermentation was conflicting. Cellulose was known to ferment, to some extent,[58,61] but so-called "ligno-cellulose" was said to be resistant.[37] One author held that grease would decompose anaerobically,[60] while another stated that it would not ferment to any substantial extent.[85] The addition of lime to favor methane production was preferred by one group of workers and opposed by another.

Of the earlier systematic studies of this fermentation, those of Söhngen were the most extensive.[73] More recent studies are those of Fowler and Joshi,[37] Sen, Pal, and Ghosh,[69] Fischer, Lieske, and Winzer,[34] and Buswell, et al.[18]

Characteristics of Methane Fermentation

MIXED CULTURES

The anaerobic fermentations, as carried out for the production of methane, differ in many respects from other types of fermentations. The most important difference is perhaps the fact that it is not necessary to use a pure culture of organisms nor is it required to maintain "purified" cultures for inoculation or reinoculation. The bacteria which are capable of producing methane are found almost universally in nature, although in preponderant numbers in mud and decaying matter. Under proper conditions, these bacteria can be cultivated to a high degree of activity within a few days. The culture can then be maintained at this activity level indefinitely, if a few simple rules, concerning chemical and physical environment, are followed. In fact, microscopic and subculture studies of this fermentation reveal such a mixed and variable flora that it appears at present to be environment rather than flora which determines the results obtained.

Continuous Process

This situation makes possible the continuous operation of the fermentation, a procedure which is quite unusual in fermentology. It is possible to carry out this process in apparatus arranged to allow the substrate to enter continuously at one point and the exhausted or inert residue to be discharged continuously at another, while the products, methane and carbon dioxide, are given off at a steady rate. There is apparently no limit to the size of apparatus which can be used. Large tanks, yielding several hundred thousand cubic feet of gas a day, operate as smoothly as laboratory-size flasks.

Independent of Substrate

Another characteristic of these fermentations is that practically any sort or kind of organic matter may be used as a substrate. Nearly a hundred different pure substances[1,80,81] and some thirty or forty natural plant and animal products,[6,7,16] such as cornstalks, milk whey, etc., have been used successfully as fermentation material. There is apparently no decomposition of mineral oils and lignin, when isolated, is not attacked at all or with difficulty.[9,49,57]

Quantitative Yields

The nearly quantitative yields of the two simple products, carbon dioxide and methane, are somewhat unique. It is true that in the various commercial fermentations of grains, the starch is practically quantitatively recovered in the products, but the fats, proteins, and fiber are not attacked at all. The methane fermentation converts the entire grain, with the possible exception of a small amount of fiber, to carbon dioxide and methane within 24 to 72 hours. The reaction is an oxidation-reduction involving water. If the composition of the substrate is known, the yield of gas can be calculated from the following equation:

$$C_nH_aO_b + \left(n - \frac{a}{4} - \frac{b}{2}\right) H_2O \longrightarrow \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right) CO_2 + \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right) CH_4$$

With a little care, it is possible to get 95 to 100% agreement with this equation. The uniformity of the end products, carbon dioxide and methane, was at first puzzling, but a study of the energy[80] of the reaction of various compounds with water showed the maximum free energy when the final products were carbon dioxide and methane.

In the experience of the author, neither carbon monoxide nor higher hydrocarbons have been observed among the products. A few earlier reports in the literature stating that ethane was formed have not been verified and the amounts reported are within the limits of analytical error in the experiments cited. However, in a private communication from Dr. John B. Davis, Magnolia Petroleum Company, Dallas, Texas, Dr. Davis has stated that very recent work, involving special techniques in concentration, and analysis by means of the mass spectrograph has detected minute quantities of ethane, ethylene, and acetylene, and even smaller quantities of propane and propylene, in the fermentation gas. The range of concentration is 0.1 to 7 ppm by volume for these various hydrocarbons.

Wide Temperature Range

In this fermentation, there is no narrow optimum temperature range. The rate of fermentation increases with increase in temperature from about 0°C to 55°C or a little higher. Maxima have been reported at 26°, 37°, and 50° to 55°C.[30] These maxima are not very pronounced and have not been noticed by some observers.

Although this fermentation can be carried out over a wide range of temperature, it may be upset by sudden temperature changes. A drop of 5°C from the temperature at which the culture has been working will frequently arrest the production of gas without materially affecting the production of acids. This may result in a dangerous accumulation of acids.

Control of Methane Fermentation

CONDITIONS

The high yields, uniformity of products, and successful inoculation and cultivation are dependent on four conditions.

1. Large Surface. The organisms seem to require a certain amount of surface for their propagation. When material of a fibrous or granular nature is fermented, this requirement is satisfied by grinding or shredding the substrate, but when dissolved substrates are employed, some sort of inert surface must be supplied. Breden and Buswell[10] used 25 g shredded, washed asbestos per l of effluent. Fischer, Lieske, and Winzer[34] found ferric hydroxide or ferrous sulfide useful for the same purpose. With such techniques, many soluble substances have been used as substrates and over considerable periods of time. Successful inoculation can be made by transferring single fibers of asbestos[10] from an active culture to a flask or test tube containing sterile asbestos and culture medium.

2. Volatile Acids. The volatile acids,[21] which are intermediates in the decomposition of higher compounds, must not exceed a predetermined value, usually 2,000 to 3,000 ppm (calculated as acetic). If the volatile-acid value is allowed to rise much above 2,000 ppm (as acetic) gas formation drops off, the quantity of acids increases rapidly, and, usually within 24 to 48 hours, all fermentation ceases.

Volatile-acid levels are followed in the fermentations by daily determinations by the method of Duclaux,[30] after acidification of the sample with phosphoric or sulfuric acid. This procedure gives a value which includes both the free acids and their salts and is independent of the pH value. It is somewhat surprising to encounter such a situation, especially since in many fermentations pH is an almost all-important factor in fermentation control. Since it is the total of volatile acids, plus their salts which controls the methane fermentation, the addition of alkali is of little use. In fact, the production of acid is stimulated in some cases by the addition of lime.

There is only one way to limit the accumulation of volatile acids. That is to limit the rate at which the substrate is added to the fermentation vessel, so that the acids will be fermented to methane and carbon dioxide as rapidly as they are formed from the raw substrate. Many of the early failures were due to the "batch" type of fermentation in which too much substrate was present.

If a culture has developed too much acid, the only remedy is dilution. This has been adapted to a series or stage digestion for certain industrial wastes.[13]

3. Scum. The formation of considerable amounts of scum must also be avoided. This is sometimes encountered when fermenting greasy material.[53] The objection to scum is that it constitutes a zone of high substrate concentration in which acids are likely to accumulate. In laboratory fermentations, this can be avoided by mechanically breaking up the scum. In plant-scale fermentations, the power required for a scum breaker is too great. Moistening the scum with liquid pumped from beneath it is a very effective and inexpensive remedy.[11] Unpublished studies show that recirculating the gas from the collecting dome, discharging it at several points beneath the scum, has distinct advantages in scum control.

4. Fibrous Material. Fibrous materials, such as paper, shredded cornstalks, etc., form a tough mat at the top of the fermentation vessel.[64] The objection to this mat is, again, that it favors the accumulation of large amounts of acid. A mat cannot be broken up mechanically nor by the circulation of liquor with any success. It is necessary to provide the fermentation vessel with suitable connections so that it can be operated alternately in an upright and an inverted position. In this manner, the mat is broken up after each inversion by the fermentative action itself. A fermentor containing a slowly rotating drum has been developed for large-scale operation.[15]

EFFECTS OF SALTS

Rudolfs,[63] and Rudolfs and Zeller[66,67] have reported a retarding effect of sulfates and chlorides in concentrations of 500 to 5,000 ppm, in batch experiments with sludge digestion. However, it was stated that, if sufficient time was allowed, digestion proceeded to approximately the same extent as in the controls. Recently Buswell, Pagano, and Sollo[22] have shown that sodium sulfate, in concentrations of 4,000 ppm or more, makes the methane fermentation of glucose at 55°C impossible. Acetic acid, however, can be readily fermented in the presence of as much as 10,000 ppm of either sodium chloride or sodium sulfate at 37°C.

Recently, Buswell and Pagano[21a] have found that nitrates in the amount of 50 ppm (calculated as nitrogen) will completely inhibit methane fermentation.

LOADINGS

It should be pointed out that the difficulties mentioned before are not met with in the ordinary plants for the reduction of municipal wastes, except at times of starting new plants or if the supervision is inexcusably bad. The digestion or fermentation tanks are so large in proportion to the load that volatile acids seldom accumulate and any scum which may form may be allowed to accumulate for a year or more without seriously interfering with the operation of the tank. It is common practice to design municipal plants with 50 cu ft of digester capacity for each pound (3 cu m per kg) of organic matter which is to be fermented a day.

The writer uses loadings of 0.2 lb per cu ft (3 kg per cu m) per day to 1.0 lb per cu ft (16 kg per cu m) per day quite successfully and continuously. It is necessary, however, to observe the rules laid down previously.

Quantitative Research Techniques

The apparatus and experimental procedure for quantitative studies of the methane fermentation were described by Buswell et al.[18] as follows:

> Inverted bottles or large filter flasks were used for these experiments. The setup shown in Figure 40 was connected to a gas collector and gasometer. Inoculation for these tanks was, at first, liquor from anaerobic digestion tanks; later, liquor from one experiment was used as the inoculum for another.
>
> The amount of substrate fed was regulated so that the weight of gas produced between feedings was equivalent to not less than 85% of the theoretical yield. At the same time that the substrate was fed, inorganic nitrogen, in the form of ammonium hydroxide, was added at the rate of 6 mg of nitrogen per gram of substrate.

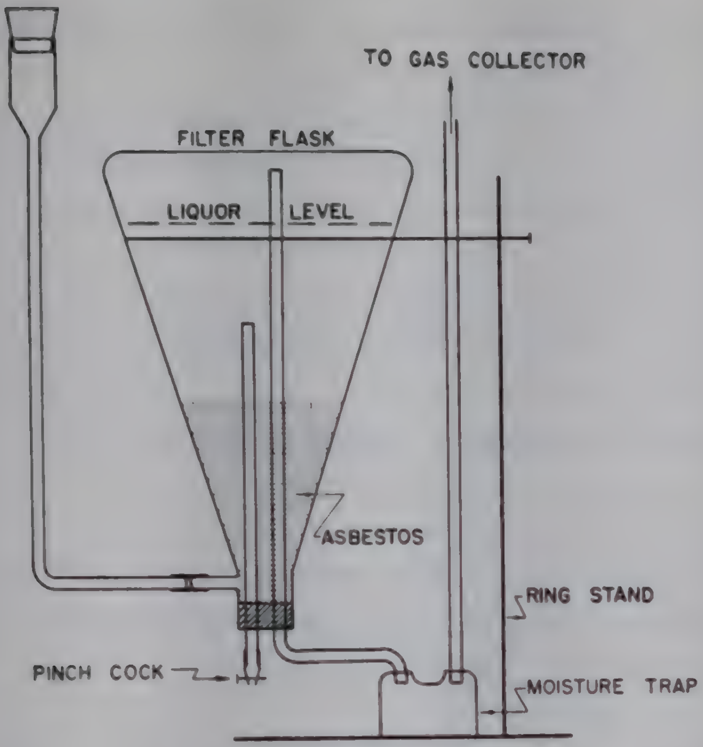

FIGURE 40. *Apparatus for Fermentation Studies*

The concentration of ammonia nitrogen in the liquor was maintained at 400 to 600 mg per liter.

Sludge containing high amounts of organic matter introduced a large possible error into the carbon balances. To obviate this difficulty and still provide a resting place for the bacteria, finely chopped asbestos[10] (previously ignited to remove volatile matter) was added to the flasks in amounts of 25 g per l of solution. To each liter of inoculating solution, 100 mg of each of the following salts was added: K_2HPO_4, $Mg_3(PO_4)_2$, $(NH_4)_2SO_4$.

For all inoculating media, the following were determined: residue, volatile matter, total carbon dioxide, ammonia nitrogen, organic (Kjeldahl) nitrogen, volatile acids (reported as acetic acid), lactic acid, chlorides, and alkalinity. At the end of an experiment, the same determinations were made on the culture medium. Analysis of the liquor was also made for unfermented substrate and qualitative tests were carried out for ethyl alcohol and formic acid. The asbestos was analyzed to determine total increase in dry weight, total increase in volatile matter, and gain in total organic nitrogen. The gas was analyzed every few days. From these data, it was possible to strike a balance and account for the amount of substrate fed but not converted to carbon dioxide and methane and the amount of carbon dioxide that was dissolved in the solution, as well as for the gas formed.

The usual laboratory incubator will house only two fermentations if carried out in apparatus of this size, even though the gasometer is placed outside with connections running through the wall of the incubator.

A smaller digester[22] (Figure 41), consisting of a 250-ml extraction flask with a brine-displacement gasometer fastened above the digester, has been designed recently. A glass tube of 14-mm outside diameter and 10 mm long is sealed to the side of the flask. This is closed with a serum stopper, through which, by means of a hypodermic syringe, samples are taken and additions are made. Fitted with stoppers and a tube for gas flow, a calibrated tube of 48-mm outside diameter is fastened above the flask. Above this is set another reservoir for the displaced brine.

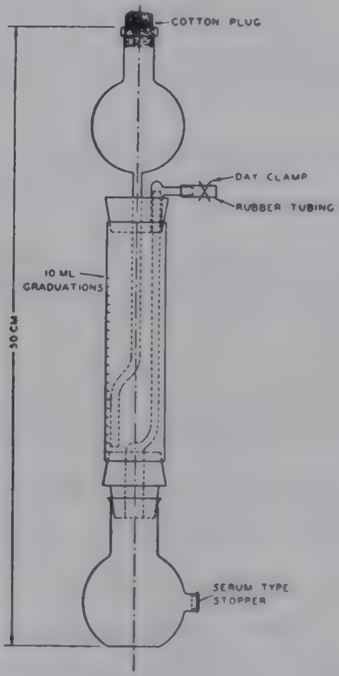

FIGURE 41. *Small Fermentation Vessel*

Gas production is observed directly by the position of the brine level in the calibrated tube and this level is easily reset to zero by opening a Day clamp at the top of the tube and releasing the gas to the air. The digester is operated with an effective volume of 200 ml and the capacity of the gasometer is roughly also 200 ml. The only disadvantage of the apparatus observed in several months' operation is that it is top heavy, and care must be taken to avoid accidents during manipulation.

These digesters could, of course, be constructed on an even smaller scale if the fermentations were not carried out on a quantitative basis. The minimum volume compatible with accuracy in gas measurement and analysis of the medium was considered to be 200 ml. Accuracy in gas measurement with this design is ±2 ml and errors are less than 2% when the daily gas production is over 100 ml. Up to 50 ml of the fermentation medium may be withdrawn daily for analysis without disturbing the fermentation.

Products of Methane Fermentation

GAS YIELDS

The actual gas yields[14] range from 12 cu ft per pound decomposed (700 ml per g) for protein to about 20 cu ft per pound decomposed for fats (1,250 ml per g). From the loadings listed before, it is seen that the ratio of the volume of gas yield to the volume of fermentor varies from 1:1 to 4:1 in most cases. In laboratory experiments, as much as 10 l of gas was obtained per day per liter of fermentor volume.

HYDROGEN FORMATION

Apparently, hydrogen is the only combustible gas other than methane which is formed by fermentation. Occasional reports of ethane may be found in the literature and carbon monoxide has also been mentioned. The amounts have been so small that when the limits of accuracy in even the most careful gas analyses are considered, it is very doubtful whether the data have justified the conclusions in regard to the presence of the last two gases.

Hydrogen is not formed in detectable amounts during the methane fermentation of the lower fatty acids, although it may well be an intermediate. When carbohydrates are used as substrates, the hydrogen yield may be as high as 30% by volume,[81] although with proper control it can be held to about 1%.

There appear to be only two types of fermentations yielding hydrogen which have been subjected to quantitative investigation. One is the fermentation of sugars by the coli group of organisms and the other the butanol-acetone fermentation.

Extensive quantitative data on the coli fermentation have been given by Rogers, Clark, and Adams.[62] Their work indicated that 30 to 50% of the carbohydrate was decomposed and probably not more than a few per cent occurred as gas. Three types of fermentation reactions were encountered. The one giving the highest yield of gas produced carbon dioxide and hydrogen in the ratio of about 2:1. The second type produced about half as much gas with a $CO_2:H_2$ ratio of 1:1. The third type gave carbon dioxide only in a relatively low yield. The second type has been investigated by Scheffer (reported by Kluyver[45]) who found about 10% gas having a $CO_2:H_2$ ratio of 1:1.

A discussion of the butanol-acetone fermentation is given in Chapter 11 of Volume 1. In this fermentation, as carried out commercially, a yield of 1.5 lb of mixed carbon dioxide and hydrogen is obtained for each pound of mixed solvents produced.[38] These gases may be utilized to produce methanol by catalytic synthesis.

Mechanism of Methane Fermentation

References on the mechanism of the bacteriological degradation of cellulose and cellulose derivatives, and especially those dealing with the anaerobic fermentation of these substances, are scarce and far from enlightening. Some information may be found in the periodical literature and in texts, such as Stephenson's[78] *Bacterial Metabolism.* For a review of the earlier literature which covers not only the bacterial degradation of cellulose and cellulose materials but also the decompositions attributed to other organisms, the reader is referred to Thaysen and Bunker's book, *The Microbiology of Cellulose, Hemicelluloses, Pectin, and Gums,*[83] and to Thaysen and Galloway's *The Microbiology of Starch and Sugars.*[84]

Since acetic acid is always found during methane fermentation, one is inclined to regard it as an important, if not universal, precursor of methane. Three possible mechanisms for the methane fermentation of acetic acid are suggested by the findings and reasoning of previous workers in this field. From the work of Omelianski[58,59] and Söhngen,[72,73] a preliminary decomposition of the acetic acid to hydrogen and carbon dioxide would be expected, with subsequent reduction of carbon dioxide to methane by the hydrogen. Barker's work[2] indicates that carbon dioxide would be directly reduced to methane and the acetic acid oxidized to carbon dioxide. The reasoning of Buswell and Neave[21] leads to simple decarboxylation as the mechanism.

The first mechanism was based on the similarity of the hydrogen and methane fermentations, but the low concentration of hydrogen found in the gas from the methane fermentation of acetic acid is evidence against this mechanism. The data of Symons and Buswell[80] show that only 3.5 l of methane was formed over a period of 100 days with regular circulation of hydrogen and carbon dioxide through a culture of 2.0 l total volume. In the same time, such a culture fermenting acetic acid could be expected to form 75 l of methane.[18,81]

The second mechanism avoids this weakness by implying a direct reduction of the carbon dioxide, without the intermediate stage of free hydrogen. This mechanism seems rather indirect and involved, but parallels that found by Barker in the fermentation of alcohols.

The last mechanism appears to be the simplest and most direct. A similar reaction *in vitro* is the chemical decarboxylation of sodium acetate with sodium hydroxide. The internal oxidation-reduction could be effected by the transfer of the hydrogen atom from the carboxyl to the methyl group. Evidence against the decarboxylation mechanism is found in Thayer's work.[82] He reasoned that if the reaction was a decarboxylation the fermentation of propionic and butyric acids should yield ethane and propane. However, in the fermentation of these acids, he found no hydrocarbon other than methane. This has been confirmed in all work where the gases have been analyzed. Therefore, if this mechanism of simple decarboxylation is to be accepted for acetic acid, it must be considered a special case, not applicable to the higher fatty acids.

Buswell, Fina, Mueller, and Yahiro[19] further investigated the action of enrichment cultures on propionates having carbon atoms 1, 2, and 3 labeled, respectively. The distribution of the radioactivity in the evolved gases, carbon dioxide and methane, indicated that propionate is decomposed in such a manner that both gases may arise from all three carbons. Carbon dioxide is, in part at least, a precursor of the methane formed. A preliminary hydrolysis of propionic acid to ethyl alcohol and formic acid is consistent with the results obtained. This was partly confirmed by simultaneous adaptation experiments. When labeled carbon dioxide was administered to cultures stabilized on ethyl alcohol and formic acid, respectively, labeled methane was found in both cases. Stadtman and Barker[76] have extended their studies on the fermentation of various lower fatty acids with C^{14} labeled substrates. Using highly purified, methane-producing cultures, capable of carrying out the incomplete oxidation of fatty acids, they were able to show that a beta oxidation is involved in the decomposition of butyric, caproic, and valeric acids. Propionic acid was shown to be oxidized in such a way that carbon dioxide is derived from the carboxyl carbon, and that acetate is formed from the alpha and beta carbons. In each of these anaerobic fermentations, methane was derived entirely from the carbon dioxide.

Stadtman and Barker,[77] using C^{14} labeled compounds, were also able to show that in the fermentation of methyl alcohol, methane, as in the case of the acetate fermentation, is derived chiefly from the organic substrate and that the reduction of carbon dioxide is of little importance.

Barker, Ruben, and Kamen[4] found evidence for the reduction of carbon dioxide in the fermentation of acetic acid through the use of $C^{11}O_2$, but they stated that the radioactive methane found might have been due to the presence of methanol carried over with the inoculum. It should also be mentioned that these workers were using a pure culture of *Methanosarcina methanica* and that even if methane should be formed by reduction of carbon dioxide in that case, this might not be the predominant mechanism in the usual methane fermentation where a mixed culture is used.

This question of the mechanism of fermentation of acetic acid is of more than academic interest. Culture failure is almost invariably accompanied by, or preceded by, the accumulation of high concentrations of volatile organic acids, largely acetic. Thus, any information concerning this mechanism might lead to methods of treatment or operation which would alleviate or prevent this accumulation of acids and possibly the failure of many cultures.

It may be seen at once that if the carbon of the carbon dioxide were marked isotopically, this question of mechanism could be settled. If either of the mechanisms involving reduction of carbon dioxide were involved, the methane produced should be similarly marked. If the reaction were a simple decarboxylation, the methane should not be so marked.

Buswell and Sollo[24] have reported that by comparing the activity of the methane and the carbon dioxide, when using C^{14} labeled carbon dioxide with acetic acid for methane production, it was shown only a very small portion of the methane was derived from the on dioxide. By studying the relation of the amount of methane ied by reduction of carbon dioxide to the time of incubation, relation was found which was interpreted as indicating a slow ction unassociated with the general fermentation, such as the ation of cell substance and subsequent autolysis. From this, it concluded that the acetic acid was fermented entirely, or very ly so, without reduction of carbon dioxide and that the ane is predominantly derived from the acetic acid and not carbon dioxide.

Stadtman and Barker,[75] in a series of parallel experiments g C^{14} carboxyl- and methyl-labeled acetate and labeled carbon ide, confirmed this observation and showed that the methane rived mainly from the methyl group of the acetic acid, whereas arbon dioxide is derived mainly from the carboxyl group. But n using ethanol as the substrate, together with labeled carbon ide, these authors reported that 82 to 100% of the methane from the carbon dioxide.

Bacteriology of Methane Fermentation

Methane, as a product of a wide variety of bacterial fermenta- processes, has been recognized for many years.[28-30] A partial f fermentations yielding methane would include composting of , leaves, and manure; retting of flax; the decomposition of lizer in the soil; the liquefaction of cellulose which causes s in various textile industries, such as paper manufacture, etc.; the production of various fatty acids from starch, sugars, and lose. Methane is one of the early products in spontaneous bustion.

Although attempts were made to isolate the bacteria respon- for this reaction very early in the history of bacteriology, or 50 years ago, none was successful in producing actual pure res until within the last 15 years. Many experimenters, in- ng the writer, had been successful in producing cultures of high activity, but microscopic examination always revealed xed bacterial growth.

Most experimenters reported two somewhat different rod- d forms and two distinctly different spherical or coccus forms. two coccus forms were of different size; one was usually seen in groups of four or eight, resembling *Sarcina*. These organisms are rather large, i.e., 4 or 5 μ in diameter. The other coccus form is very small, being something less than 1 μ in diameter, and is referred to as a *Micrococcus*. The rod forms are similar, being rather long and thin and slightly bent. The distinguishing characteristic is a tendency of one of them to form long filaments.

Two new species of the methane-producing bacteria were recently obtained by Stadtman and Barker.[76] These organisms differ from the methane-producing bacteria previously described in that they are able to carry out the incomplete anaerobic oxidation of fatty acids.

The methane organisms, in addition to producing methane, also have the following common characteristics: They are non-motile, non spore bearing, and Gram negative. They are obligate anaerobes and are capable of using ammonia as a source of nitrogen. They all develop at a relatively slow rate, requiring several weeks after inoculation for the development of an active culture.

FIGURE 42. *Bacterial Flora from Jefferson Junction Plant; numerous* Micrococcus *forms; fermentation rapid*

The earlier workers found that many of the organisms could produce methane from ethanol or calcium acetate. Barker,[2,3,1] using a medium in which the only source of food was ethanol, was successful in isolating a pure culture of one of these four types of organisms. This was the *Methanobacterium omelianski.* Recently, in the author's laboratory, there was obtained what appears to be a pure culture of the large *Methanosarcina* form, but this is incapable of producing methane from calcium acetate.

The relation of bacterial flora to successful operation is well illustrated by Figure 42. This sample was taken from a plant after it had been in successful operation for a little over a year and when it was producing gas at a very high rate. It will be noticed that this figure shows a very nearly pure culture of a *Micrococcus,* one of the organisms observed previously by practically all workers in methane-producing fermentations.

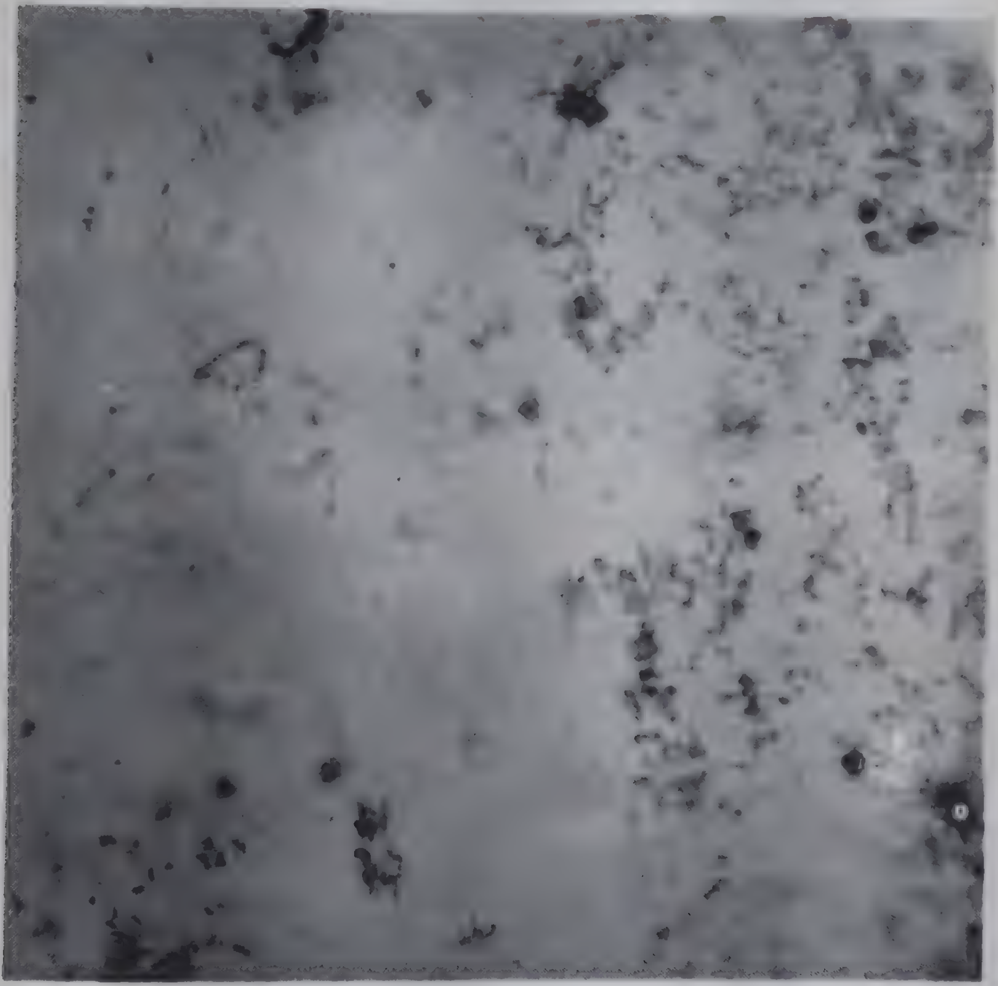

FIGURE 43. *Bacterial Flora from Crystal Lake Plant; mixed flora; fermentation poor*

Unfortunately, a comparable figure at the time of poor operation of this plant is not available. Figures 43 and 44, however, made from contents of a fermentation tank at Crystal Lake, Illinois, reflect operating conditions very strikingly.

Figure 43 shows relatively few organisms and no particular predominance of any one methane-producing form. At the time this sample was taken, the fermentation was very slow, producing only about 100 cu ft of gas per hour.

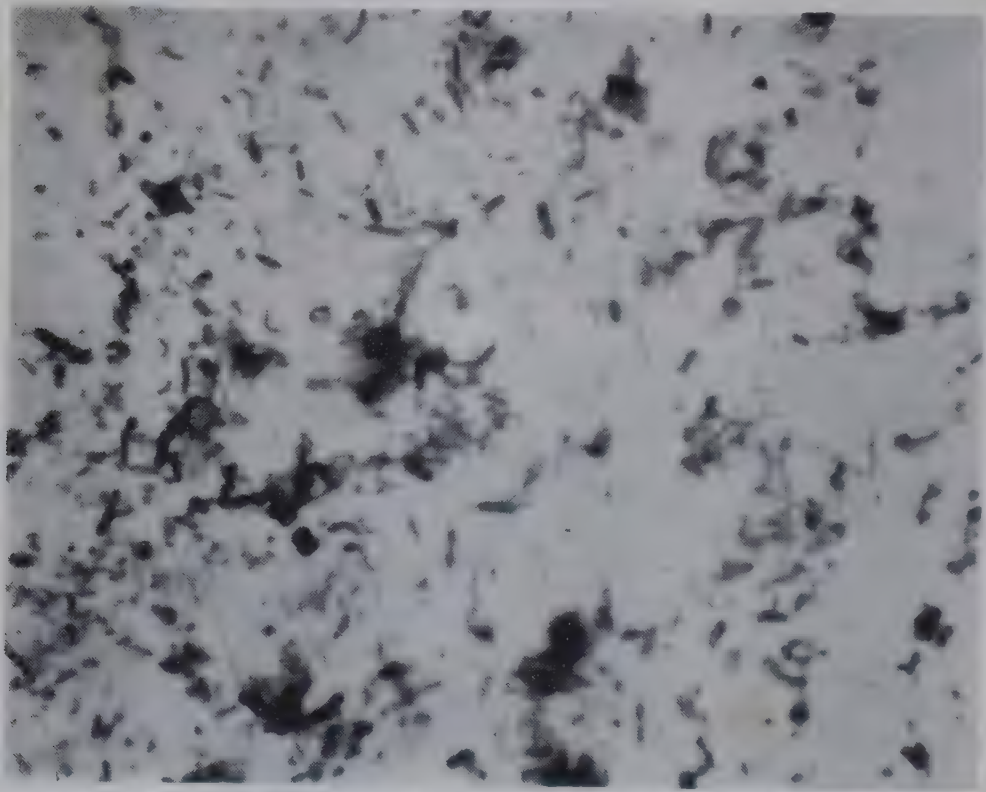

FIGURE 44. *Bacterial Flora from Crystal Lake Plant; mixed flora, methane producers predominating; fermentation good*

Figure 44 shows a luxuriant growth of typical methane-producing organisms, particularly the *Micrococcus,* some of the rods, and some of the large *Sarcina.* Careful control of temperature, feed rate, and circulation had increased the gas yield to about 1,000 cu ft per hour.

Figure 45 shows a greatly enriched culture grown in laboratory flasks with calcium acetate as a source of food. This photograph shows a large number of the large *Sarcina* forms and a considerable number of the small *Micrococci.* The long threads are asbestos fibers which were added to the culture to provide the mechanical support which has been found necessary for rapid growth.

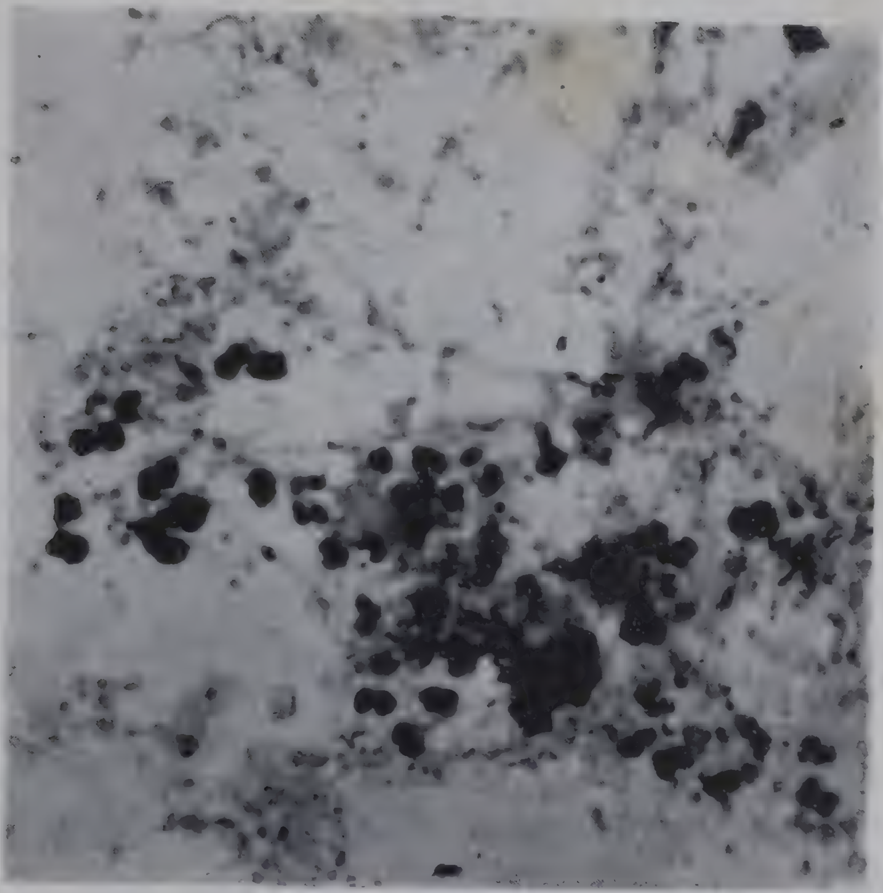

FIGURE 45. *Laboratory Methane-Producing Culture; filaments are asbestos fibers*

MICROBIOLOGICAL TREATMENT OF MUNICIPAL AND TRADE WASTES

General Considerations

The term "municipal wastes" as used here applies to sewage and the term "trade wastes" applies to the water-carried wastes discharged by manufacturing plants. Certain biological processes will be discussed, which, broadly speaking, are applicable to the stabilization of all organic wastes before they are discharged into a stream. A few of these waste liquors carry mineral salts or acids which may be responsible for more or less damage to the stream. A great majority of these wastes contains organic matter of biological origin which, on decomposing, causes putrefactive odors and a depletion of the oxygen of the stream below the requirements of normal fauna and flora. It is obviously this second class of wastes with which we are primarily concerned in the discussion of biological processes in industrial-waste treatment.

The primary object of treatment is to prevent oxygen deficiency in streams. Oxygen deficiency is the basic cause of nuisance and destruction of fish and other aquatic life, including normal vegetable life. The elements carbon and hydrogen and, to a small extent, nitrogen are the chemical factors responsible for consumption of dissolved oxygen. Therefore, the problem is to remove substances containing unoxidized compounds of these elements.

Various bacteria are capable of accomplishing this by forming either (a) relatively insoluble gases or (b) insoluble solids, from the carbon in the organic matter. These processes might be classified as fermentative or gas producing and precipitating (bioprecipitation), respectively. A more common classification of the processes for biological stabilization is made on the basis of the classes of bacteria and other microorganisms which are responsible for the results produced. This classification divides the stabilizing processes into the aerobic and anaerobic groups; that is, those produced by organisms requiring air or oxygen and those carried on in the absence of oxygen.

Except in the case of very simple substances, like acetic acid or glucose, aerobic and anaerobic actions both yield solid and gaseous end products under practicable conditions. When carried to an extreme, the solids may be almost, if not quite completely gasified by both processes.

The character of the solids and gases is distinctly different in the two cases. The aerobic process naturally yields carbon dioxide the hydrogen forming water. The solids produced are largely microbial protoplasm, together with colloidal and suspended matter[17,23,26,42] caught by the biological jelly. The solids are 60 to 70% of the original material, the remainder going to carbon dioxide and water.

The anaerobic process is essentially a methane fermentation and yields a humuslike solid, while the gas evolved is composed of methane and carbon dioxide, with small quantities of hydrogen. The methane and carbon dioxide together usually amount to 95% of the total gas collected, and the ratio of $CH_4:CO_2$ varies from 1:1 to 3:1, depending on the composition of the waste decomposed and to a lesser extent, on the conditions under which the fermentation s carried out.[6,7,9,54,80,81] The humuslike solid remaining at the end of active anaerobic fermentation may amount to 40% of the original when woody material is decomposed, while relatively simple and ess resistant substances, such as sugars, fats, and certain proteins are completely gasified.[7,20,21,27,47]

Data in the literature cited indicate that, with the exception of hydrocarbons, practically all carbonaceous substances can be quantitatively converted to methane and carbon dioxide if fermented long enough and under proper conditions. Even lignin yields slowly to this action.[9]

Both the aerobic and anaerobic processes effectively accomplish the stabilization of putrescible waste liquors. The choice between the two will depend on the particular problems involved. Where conditions permit its use, the anaerobic process is much cheaper per pound of organic matter stabilized. A good example is the calculation made in the author's laboratory[20] of the relative cost of treating dairy wastes by anaerobic fermentation and on trickling filters.

The anaerobic process requires simple covered tanks of suitable design. Assuming an average dosage of 1/25 of a volume of milk waste (undiluted basis) per day, per unit of tank volume, it would require a tank, or tanks if operated as a two-stage process, of 5.72 cu ft capacity for the anaerobic fermentation of milk waste containing 1 lb dry weight of milk waste solids. At 50¢ per cu ft, this amounts to only $2.86 per pound of milk solids treated. This fermentation would remove at least 95% of the pollution load. The remaining 5% contained in the overflow liquor could be stabilized readily on filters. Assuming that this final treatment could be made at a cost similar to that given by Kimberly[44] for filter treatment of milk wastes, the total investment for complete treatment would be $8.70 per pound of solids if trickling filters were used following the anaerobic digestion, or $17.46 per pound if sand filters were used, as compared with $116.80 per pound if trickling filters were used alone, or $292 per pound if sand filters were used alone. These igures are not given to show actual costs, but rather the relative costs of the two processes.

While the anaerobic process is much less expensive, it has wo serious limitations. First, for reasons as yet obscure, it is impossible to produce a final effluent comparable to that yielded by erobic treatment. The discharge from anaerobic fermentation is usually dark colored and rather foul with a b.o.d. (biological oxygen demand) of several hundred. Where a high dilution is ava able, this is not a serious handicap and where heavy untreate wastes are being discharged, the 75 to 90% reduction in the b.o.d load effected by anaerobic treatment is such a great improvemen that it may be adopted as a preliminary measure. As will be described in more detail later, the anaerobic process may be used a a preliminary step followed by the more expensive aerobic proces where necessary. The second limitation is that the cost of anaerobi treatment increases as the concentration of the waste decreases This is due to the large tankage required to accommodate a give number of pounds of organic matter when dilute liquors are to b handled. It has usually been found that the economic advantage of the process disappear when wastes containing less than 1% dr weight of organic matter are to be treated. There is no upper limi of concentration to this process. However, when the concentratio of the waste reaches 8%, it can usually be evaporated and dried at cost which permits its profitable sale as a stock feed or as a fertilize

Commercial Development of Anaerobic Methane Fermentation

The commercial application of this fermentation dates we back into the last century when it was employed merely to stabili and humify organic wastes (i.e., in septic tanks).

It was not until 1897 that a waste-disposal tank serving a lep colony in Matunga, Bombay, was equipped with gas collectors an the gas used to drive gas engines.[36] At about the same time, th waste-disposal tanks at Exeter, England, were partially equippe with gas collectors and the gas was used for heating and lightin at the disposal works. In 1911, a company was formed in Australi for producing and using fuel gases which resulted from the bi logical decomposition of municipal wastes. Kessener of the Hagu Netherlands, in 1914, described seeding and culture methods f optimum gas yields from straw-board wastes. In the United State in 1915, Hommon[42] equipped waste-treatment tanks with gas c lectors and used the gas. In 1920, Watson[59] of Birmingham, En land, reported a study of methane production from sludge digestio and called attention to the fact that a considerable amount methane can be produced in this way. Following his suggestion, th

disposal plant which was put into operation in 1927 by his
ssor, Whitehead, was equipped with gas engines that are
ated on the gases produced from sludge digestion. This use
e gas cuts down very materially the operating cost of the dis
l works. In the meantime (1925), Imhoff in Germany had
pped the sludge-reduction tank in Essen with gas collectors and
ected them to the city mains. The gas was found satisfactory
general municipal use and sold to the city. In the same year,
vell and Strickhouser[32] observed that the sludge-reduction tanks
ecatur, Illinois, were producing about 200,000 cu ft of gas a day.
s large yield is due to a considerable amount of wastes from
rch works which are discharged into the city drainage system.
average yield at Decatur was about 125,000 cu ft of gas a day.
At present, the use of gas for power or heat at municipal dis-
l plants is almost universal in many countries. Fischer[33] re-
ed in 1946 that fuel and fertilizer from sewage was the aim of
man treatment plants. Digester gas was developed as a motor-
cle fuel to replace gasoline. The purified gas contained 90 to
methane and about 135 cu ft of this gas at atmospheric pres-
was equivalent to 1 U.S. gal gasoline. Its cost was about
not considering fixed charges on equipment and facilities.
m[34] in 1948 stated that in a conventional-type sewage-treatment
t, with separate sludge digestion, the digester gas is used in a
ngine to supply pumping power, saving the plant $100 a month
uel bills. The dried sludge is sold for orchard fertilizer and the
rinated plant effluent is used for irrigation. A private communi-
n from the superintendent, C. T. Wilson, disclosed that in the
ndar year of 1949, the disposal plant of the city of Waterloo,
, received $47,000.29 for gas, besides providing all the heat
about the plant. A recent communication from the manager,
. Pray, stated that the city of Fort Dodge, Iowa, has a 250-hp
ngine driving a 175-kw generator, the fuel for which is methane
produced at the sewage-treatment plant. Disposal of surplus
ric power is made to the local power company under an inter-
ge of power contract.

In addition to the general use as fuel, gases from sludge-diges-
plants can be also utilized for chemical manufacture. Wilson[35]
ibed the use of digester gas as raw material for the production
rmalin in an English plant. In this process, all sulfur com-
ds are removed from the gas, water vapor is added, and the

methane-carbon dioxide mixture is passed over a granular nickel catalyst at 950°C to produce carbon monoxide and hydrogen. Treatment of these gases at 2,500 psi pressure over a zinc chromite catalyst converts them to methanol, which is then dehydrogenated to formaldehyde.

An interesting possible application of this fermentation is that proposed by Fischer, Lieske, and Winzer,[88] namely, the detoxication of coal gas by means of the reactions,

$$\begin{aligned} CO + 3H_2 &\longrightarrow CH_4 + H_2O \\ CO + H_2O &\longrightarrow CO_2 + H_2 \\ CO_2 + 4H_2 &\longrightarrow CH_4 + 2H_2O \end{aligned}$$

Söhngen[73] found that hydrogen was formed in the methane fermentation and suggested that it was intermediate in the formation of methane. He showed that in the presence of the methane-forming culture, the reaction

$$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O$$

took place quite readily. This observation of Söhngen has been confirmed in the author's laboratory.[79] The volume efficiencies have not been developed to date, but this phase of the process holds interesting possibilities.

TREATMENT OF INDUSTRIAL WASTES BY ANAEROBIC METHANE FERMENTATION

When an industrial waste is very concentrated (containing 1% or more solids) as in the case of distillery, dairy, packing-house, and other wastes, methane fermentation offers an attractive method of treatment.[5,8,12,16] The operating cost is small, although the original investment is rather large. Altogether, it appears that gas can be produced from these wastes at 5 to 10¢ per 1,000 cu ft.

Design of the Digester Fermentation Plant

The preferred design (Figure 46) for an anaerobic fermentation plant consists of two, or preferably three, tanks with floating covers arranged to operate in series. A heat exchanger is necessary to maintain the desired temperature (about 95°F for cold wastes and 130°F for hot wastes). The floating covers are conical and carry domes for gas collection. These are protected by flame traps. The bottoms of the tanks are conical, sloping toward the center. The

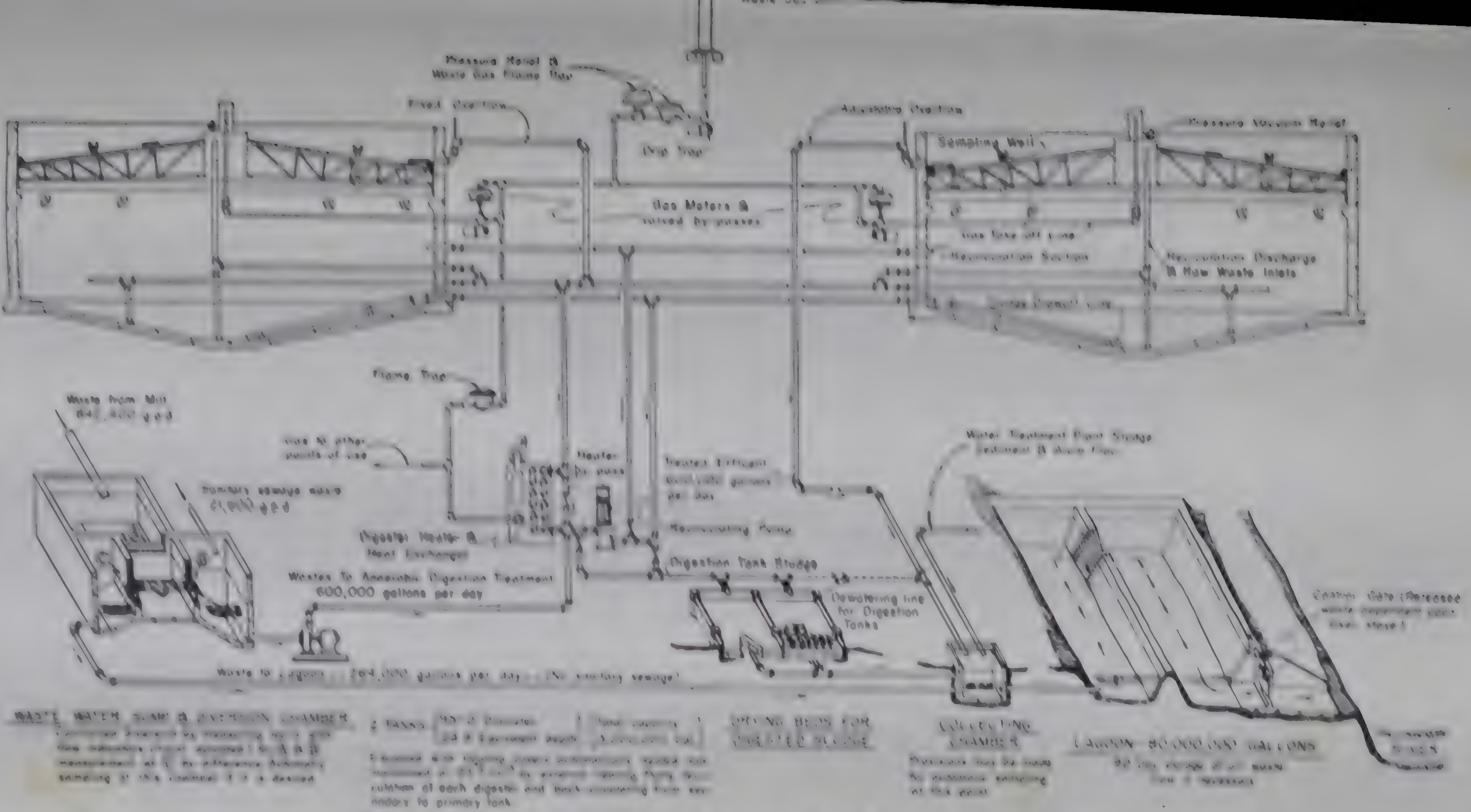
Pressure Relief &
Waste Gas Flame Trap
Fixed Overflow
Drip Trap
Adjustable Overflow
Sampling Well
Pressure Vacuum Relief
Gas Meters &
valved by-passes
Gas take off line
Recirculation Suction
Recirculation Discharge
& Raw Waste Inlets
Flame Trap
Waste from Mill
Gas to other
points of use
Heater
by-pass
Treated Effluent
gallons
per day
Sanitary sewage waste
Digester Heater &
Heat Exchanger
Recirculating Pump
Wastes to Anaerobic Digestion Treatment
600,000 gallons per day
Digestion Tank Sludge
Dewatering line
for Digestion
Tanks
Water Treatment Plant Sludge
Control Gate
Waste to Lagoon gallons per day (No sanitary sewage)
WASTE WATER SUMP & DIVERSION CHAMBER
2 TANKS
DRYING BEDS FOR
DIGESTED SLUDGE
COLLECTING
CHAMBER
LAGOON GALLONS

FIGURE 46. *Design for an Anaerobic Fermentation Plant*

circulating pumps and piping system must be laid out to provide the following variations in a three-tank system: (1) use of the series in any order, i.e., 1, 2, 3; 2, 3, 1; 3, 1, 2; or 3, 2, 1; etc.; (2) circulation of contents of each tank from bottom or center to center or top; (3) circulation of the contents of any pair of tanks or all three tanks in a similar manner.

Performance in Digester Fermentation Plants

The largest plant constructed to date is at Pekin, Ill. In a private communication, the late Mr. Almon Fales furnished the following design data:

> The weekly average volatile solids content of the four major wastes to be treated by anaerobic digestion ranged from 0.79% to 1.36% averaging 1.05% for the 93 weeks ending January 1, 1938.
>
> The average quantity of these wastes during this period was 222,300 gal per day, 6 days a week, the 6-day average for the week of maximum flow being approximately 320,000 gal per day.
>
> The digestion capacity of the six digestion tanks to be built will be approximately 285,000 cu ft not including the space reserved for gas but including an allowance of 10% for accumulation of sludge. The effective digestion capacity is accordingly approximately 259,000 cu ft.
>
> The average 6-day loading for the 93-week period, with an average of 222,300 gal per working day and an average of 1.05% volatile solids, would be 0.075 lb per cu ft per working day. An additional digestion period of 1 day per week with little or no wastes added is accordingly afforded.
>
> The loading based on the maximum average flow per working day for the week of maximum flow and the maximum weekly average of 1.36% of volatile solids, which combination would rarely if ever occur, would be 0.140 lb per cu ft per working day.
>
> I should like to point out that in the anaerobic digestion tests, we obtained excellent results with loadings as high as 0.3 lb per cu ft of digestion flask capacity after favorable biological action had become established. As a matter of fact, we originally proposed four digestion tanks having an effective total digestion capacity of 170,000 cu ft which allowed a considerable factor of safety for the loading of the large digestion tanks in practice. In order to make it possible to maintain high efficiency in case of continuous operation of the factory at capacity production, it was decided to provide six tanks instead of four, thus increasing the digestion capacity by 50% and also increasing the flexibility of operation.

TABLE 45. ANAEROBIC DIGESTION OF TRADE WASTES

Location	Pekin, Ill.	Peoria, Ill.	Crystal Lake, Ill.	Jefferson Junction, Wis.	Carthage, Ohio
Waste	Yeast (Molasses)	Butanol (Grain)	Yeast (Molasses)	Malt steep water trickling filter sludge	Distillery (Grain)
Volume flow, gal/day	222,300	300,000	160,000	2,000	500
Tank, gal	2,300,000	3,000,000	623,000	40,000	7,200
Solids					
volatile, %	1.05	3.0	0.7	3.5	3
B.O.D.					
Raw	10,000	17,000	5,000		16,000
Effluent	2,000	2,420	1,500		1,600
Removal, %	80	69.8	70		90
Loadings					
lb/cu ft/day	0.108	0.114	0.104	0.10	0.143
Gas					
cu ft/lb	5.0	9.3	4.25	9.2	11.0
vol. per tank vol.	0.525	1.06	0.43	1.0	1.5

In Table 45 are summarized extensive performance data based on several years of operating experience in five trade waste-digestion plants.

The second column shows the usual performance of the Standard Brands yeast plant at Pekin, Illinois. The purification amounts to 80% at a loading of 0.10 lb per cu ft per day. Since the third-stage tanks show practically no improvement over the second stage, the effective load is 0.15 lb per cu ft per day. This plant has been in continuous operation for 10 years.

The third column shows the performance of digesters operated by the Peoria Sanitary District while digesting the "slops" or "still bottoms" from the Commercial Solvents butanol-acetone plant. We are indebted to Mr. L. S. Kraus for these data. The digesters, which have been in operation for about 15 years, except for a few periods when the butanol plant was shut down, are accomplishing a 69.8% reduction in b.o.d. at a loading of 0.114 lb per cu ft per day.

The fourth column shows the normal operating results of a plant at Crystal Lake, Illinois, put into operation in 1944. This plant consists of a two-stage digester for handling wastes from the manufacture of yeast. The waste is slightly more dilute than that at Pekin and the loading is correspondingly less, as is the gas yield. The removal of b.o.d. is 70%. The primary digester is equipped with a floating cover and has a capacity of 866,500 gal. The secondary digester has a fixed cover and a capacity of 264,800 gal. We are indebted to Col. M. W. Tatlock of Ralph L. Wolpert Company, Dayton, Ohio, for performance data on this plant.

The data in the fifth column were obtained on an installation treating the waste steep water from a malt-manufacturing plant. The steep liquor is free from suspended solids and is discharged directly to a trickling filter. The filter "unloads" continuously and the load passes to a settling tank which is provided with mechanical sludge removal. This sludge is digested in two 20,000 gal digesters equipped with floating covers. The effluent from the digesters is returned to the influent to the trickling filter. The sludge is digested until it drains well on sand beds.

Although results in the sixth column for grain-distillery wastes were obtained on a two-stage pilot plant operated for about 4 months, they are believed to be representative of what can be accomplished under carefully controlled conditions. A purification

0% was obtained with a loading of 0.143 lb per cu ft. This t-plant experiment has been described previously in detail.[19]

Summarizing, it may be said that: (1) Anaerobic fermentation nts for the reduction of b.o.d. of organic wastes are capable of tinuous operation for indefinite periods; (2) loadings of 0.14 per cu ft per day of organic wastes have been found feasible many pilot-plant studies; (3) existing plants built with a large ty factor are loaded to 0.1 lb per cu ft per day, and regularly duce a 70 to 80% improvement in the quality of the wastes.

Recent Investigations on Anaerobic Digestion

Numerous laboratory experiments indicate the possibility of ch higher loadings than have been employed in plant installa- ns. Because of the newness of the process, commercial installa- ns have been designed with a large safety factor which experience howing to be unnecessary. Table 46 lists some of the laboratory ults with heavier loadings.

Recent literature gives information on the stabilization of er industrial wastes by anaerobic digestion, a few examples which are worthy of brief mention. Sawyer[68] gave the results of ne experiments conducted at the University of Wisconsin on the sibility of treating waste sulfite liquors by the standard biological thods. Good and uniform b.o.d. removals, varying from 93 to %, were obtained with sulfite liquor concentrations as high as %, even though very little of the original color was removed. gleton[79] reported that pilot-plant studies with anaerobic diges- n of wool-scouring wastes gave a b.o.d. reduction of 82%, volatile tter 65%, and grease 90%. Morgan[52] reported pilot-plant treat- nt of deinking wastes. Primary sedimentation for 96 hours re- ed suspended solids from 2441 to 1326 ppm and b.o.d. from 530 399 ppm. Activated-sludge treatment reduced the suspended ds to 885 ppm and the b.o.d. to 169 ppm. Trickling-filter treat- nt was not satisfactory. Rudolfs and Trubnick[65] found on the t-plant scale that two-stage digestion gave average removals of total solids, 68% volatile matter, 85% b.o.d., and 74% organic ogen with compressed-yeast wastes.

Lamb[43] reported that when preliminary study is made to deter- e the proper retention time and the amount of air necessary per on of sewage, the activated-sludge process can be satisfactorily

TABLE 46. FEEDING AND GAS–PRODUCTION RATES OBTAINED DURING THERMOPHILIC DIGESTION OF VARIOUS INDUSTRIAL WASTES

Waste	Rate of Feed		Gas Recovery	
	Volume basis, volume per day per tank volume	Solids basis, lb total solids per day per cu ft of tank	Volume basis, volume of gas per day per unit of tank volume	Solids basis, cu ft of gas per lb of total solids
Commercial Solvents' beer-slop (butyl-acetonic) While fermenting rye, 3.3% total solids, 91% volatile	0.500 0.250 0.167	0.930 0.620 0.310	7.00 4.20 3.00	7.50 8.60 9.70
While fermenting corn, 2.0% total solids, 92% volatile	0.250	0.330	3.70	11.00
Heinz distillery waste 7.1% total solids, 96% volatile, pH 4.1	0.036	0.160	2.00	12.50
Buttermilk and whey waste 7.0% total solids, 91% volatile, pH 5.0, high sugars	0.035	0.150	1.60	10.70
Artichoke waste		0.095	0.81	8.35
Extracted chicory		1.560	1.64	10.50
Sugar beet waste		0.078	0.96	12.30
Packing house paunch manure		0.370	3.10	8.95

l to wastes from canneries, packing plants, milk-processing
and acid-pickling processes, either alone or when mixed
mestic sewage. Uhlmann[87] found that activated-sludge treat
l rendering wastes, followed by lime settling, aeration, se
settling, and sand filtration, gave a b.o.d. reduction of more
5%.

TERIOLOGICAL ASPECTS OF USES OF SEWAGE

order to complete the picture and make a more rounded
tation of the whole problem of waste disposal, some of the
waste waters not covered elsewhere, and perhaps lying some-
outside of the principal purpose of these discussions, will be
ed briefly.

Sewage Farms

ne of the oldest attempts at the use of wastes is the recovery
tilizer. As is well known, excreta contains considerable
ts of available nitrogen, which is a very important plant
The Chinese probably have the most effective method for
covery of nitrogenous matter. This consists, as most of us
in the collection of excreta in dry latrines with tight con-
which makes possible the transportation of the undiluted
al directly to agricultural land. This method introduces
problems, since the consumption of fresh vegetables pro-
on lands so fertilized is a well known method of transmitting
nal diseases, including both the amoebic and bacillary types
ntery.

more modern method of removal of wastes by water carriage
ts an entirely different picture. In these systems, 1 gal waste is
diluted with 50 to 150 gal of water, so that even strong
ic sewage contains less than 0.05% of organic matter. In
words, strong domestic sewage is 99.95% pure, which exceeds
rity of a certain well advertised soap! With this high dilu-
he cost of concentration of the nitrogenous material exceeds
h value of the fertilizer. An early attempt to utilize sewage
development of sewage farms. In this case, the sewage, after
l of coarse fibrous and granular material, is distributed over
d through irrigation ditches supplying both moisture and
lant food. In arid regions, as for example, the area near

Denver, Colorado, the value of the moisture is of prime importan and the process is, in general, successful.

On the older sewage farms, such as those operated by Par France, and Berlin, Germany, the amount of moisture was som times greater than the soil required and sour and soggy lan resulted.

Another example of the use of sewage as a fertilizer is the c velopment of fish ponds in the vicinity of Munich, Germany. He carp, which depend on aquatic vegetation for their food suppl are successfully raised. Sewage, after the removal of coarser pa ticles, serves to fertilize and stimulate the growth of aquatic veg tation and, of course, as the organic material is removed from t water, purification is accomplished.

Recovery of Fertilizer

The next development in the recovery of fertilizer from se age was the introduction of purification processes which remov the organic matter in the form of sludge. This sludge, after dig tion, is drawn out to sand beds to be dried. The digestive proc liquefies a considerable portion of the nitrogenous compoun so that the sludge is relatively poor in nitrogen (1 to 3% on a d basis). This sludge contains 1 to 2% of available phosphoric ac and a small amount of potash. The principal value of this slud is, therefore, as a humus material. It is sometimes sold for a fa price where soil in the vicinity requires humus, but in many cas it is impossible even to give it away.

The introduction of the activated-sludge process resulted in much higher recovery of nitrogen, since by this process, the solub and colloidal nitrogen compounds are taken up by biological grow which later settles. This process is best described as a bioprecipi tion. Activated sludge is chemically treated and artificially dri in considerable quantities at Milwaukee and Chicago as well several other large cities. This sludge contains 6% of nitrogen, w 3% of phosphoric acid, and is, of course, of greater value. T total amount of activated sludge sold annually in the United Sta is in the neighborhood of 100,000 tons.[28]

Interesting bacteriological problems exist in connection w both the digestion of settled sludge and the production of activa sludge by bioprecipitation. These are strictly bacteriological pr nd advances in their operation have depended on studies of lora.

Reuse of Wastes

he handling of industrial wastes and the recovery of valuable cts has been handicapped by the large volume of water to be ed. Notable strides in decreasing the volume of industrial have been made in the last 25 years. Starch manufacture and ulp and paper industry are examples.

n starch manufacture, corn is steeped in water before final ing for the removal of the starch grains. According to Green- Cornell, and Hatfield,[33] the wastes originally consisted of all rn kernel, except the starch. Early in the history of the in- y, the germ was separated for the oil, and the grits and gluten were added to the oil cake for cattle feed. The remaining le sugars and proteins, dissolved from the corn in the steeping tarch-washing process, were 2 to 4% of the corn. The liquor a single steep is too dilute to permit its evaporation and the ery of the gluten. Introduction of sulfur dioxide prevents the h of putrefactive bacteria, so that this weak process water can ed in the steeps instead of fresh water and the heavy steep can be evaporated and added to the cattle feed. This pro- e closed the cycle and bottled up the process; fresh water only the process for washing the starch on vacuum filters.

n paper making, the fiber, as it goes to the paper machine or drums, is suspended in 95 to 99 parts of water. A certain nt of soluble matter is taken up and the water, after the re- of the fiber (so called "white water") serves as a medium e growth of slime-forming bacteria which eventually attack aper stock. In the last 25 or 30 years, the practice of dis- ing the white water with chlorine and treating it with some bacteriostatic compound (e.g., certain chlorinated phenols) ade it possible to reuse this white water indefinitely. Accord Skinner,[71] many paper mills now operate on this closed sys- here all, or practically all, of the water is reused. This has d in the lessening of stream pollution by white water and also ncial savings to the mills. A saving of a very considerable t of paper fiber is made since some fiber always passes through per machines. Savings of fiber easily amount to $100 per day achine.

Southgate[54] reported a substantial saving in flax retting esta
lishments by reusing the retting liquor continuously. The was
water from the anaerobic retting of flax is very polluting. Althoug
chemical treatment of these wastes has been beneficial, the necess
for large-scale waste-water treatment was avoided by reusing th
retting liquor and continuously aerating it in the retting tank.

The extremely high demand for water for industrial purpo
in the last few years has led to some very interesting developme
in the reuse of sewage. This was a subject of a symposium held t
the joint meeting of the American Water Works Association a
the Federation of Sewage Works Associations in San Francisco
1948. Veatch,[55] in this symposium, cited nine large installatio
where the purified effluent of a sewage-treatment plant was used f
such industrial purposes as steam raising, cooling, and quenchi
of steel and coke. Probably the largest such installation is th
at Baltimore and described by Wolman, where some 20 million g
lons of reclaimed sewage are used a day as an industrial wat
supply. This development is of interest to bacteriologists, since
presents a very important problem of control of the disinfecti
of such a large amount of heavily polluted liquor.

FERMENTATIVE UTILIZATION OF INDUSTRIAL WAST

Certain large industries produce tremendous quantities
wastes having high organic content, particularly of carbohydrat
which present a considerable disposal problem. Prohibition
stream pollution requires treatment of such wastes before they c
be discharged into streams. Activated-sludge or anaerobic fermen
tion plants have been installed by some of these industries to
sume the disposal load, as has been discussed before. Other ind
tries have attempted to realize some practical benefit from su
wastes by other fermentation procedures. Space does not permi
detailed treatment, but a few recent papers related to this approa
to the disposal problem may be reviewed briefly.

Nolte, von Loesecke, and Pulley[56] investigated the producti
of feed yeast and industrial alcohol from citrus-waste press ju
Torula utilis was used in the yeast production. Four differe
yeasts were used in the production of alcohol. About 25 gal of pr
juice were required to yield 1 gal of 190° proof alcohol, with a p
duction cost of 11.3¢ per gallon of alcohol. McNary[51] reported t

al problem of citrus wastes in the Florida canning indus- solved by pressing, drying the press cake as carbohydrate d, and concentrating the press juice to citrus molasses for to animal feeds. Fenstel and Thompson[32] have investi- possibility of using pear-canning wastes for yeast culture. disposal of vast quantities of sulfite waste liquor from l pulping industry has become an increasingly important The carbohydrate content of the waste liquor may be for fermentation to yield useful products. For example, aste liquor is now used commercially in a few plants for luction of industrial alcohol and of food and feed yeast apters 4 and 10 of Volume I). Tyler[86] reported that fodder- oduction reduced the sugar content of sulfite waste liquor by d reduced the b.o.d. by 32 to 56%. The alcohol fermenta- uced the b.o.d. of the sulfite waste liquor by 43%. Joseph[43] l the same reduction in b.o.d. in the industrial-alcohol plant d by the Ontario Paper Co. Other fermentations which en proposed for utilization of sulfite waste liquor are the butyl alcohol fermentation investigated by Wiley, John- Kay, and Peterson,[88] the butyric acid fermentation investi- y Daniels and McCarthy,[23] and the lactic acid fermentation ated by Leonard, Peterson, and Johnson.[47] Although these t achieved commercial exploitation, sulfite waste liquor is g increasing attention as a potential raw material for fer- on industries.

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